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Sir:

Transmitted herewith for filing is the patent application of

Inventor: JOSEPH HAMBURGER ET AL.

For : TRANSGENIC EUKARYOTIC PARASITES AS UNIVERSAL GRAFTS FOR USE IN VIVO DELIVERY OF BENEFICIAL GENE PRODUCTS IN HUMANS AND IN ANIMALS

Enclosed are:

☒ 19 sheets of informal drawing(s).

☒ An assignment of the invention to YISSUM RESEARCH AND DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM

☐ A certified copy of a ______ application.

☐ An associate power of attorney.

☒ A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27.

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Respectfully,

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APPLICATION FOR PATENT

Inventors: Joseph Hamburger and Avraham Laban

Title: TRANSGENIC EUKARYOTIC PARASITES AS UNIVERSAL GRAFTS FOR USE *IN VIVO* DELIVERY OF BENEFICIAL GENE PRODUCTS IN HUMANS AND IN ANIMALS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to transgenic eukaryotic parasites such as parasitic worms and, more particularly, to the use of transgenic eukaryotic parasites such as parasitic worms as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals. Most particularly, the present invention relates to the use of Bilharzia parasites (*Schistosoma* Spp.) as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals.

Genetic engineering for producing desired gene products is presently the sole means available for producing, rather than extracting or isolating from natural sources, moderate to large size proteins in quantity. This is especially true for proteins of therapeutic importance such as hormones, enzymes, receptors, antigens and cytokines.

The quantity required as well as the required antigenic compatibility to the recipient of these products dictates the use of recombinant DNA technologies. Introducing the desired genes to host cells *in vitro* with appropriate upstream control elements has been used for the production of medically important proteins. Recombinant therapeutic proteins initially prepared in bacteria are increasingly being produced in yeasts, and even more so in mammalian cells for obtaining suitable post-translational glycosylation where it is required for a fully functional product[1,2,3].

Most of the products of recombinant DNA technology are proteins that cannot be given orally for therapy. In general they are unstable and poorly absorbed from the gastrointestinal tract thus they must be given by injection, which limits their use.

A number of drug delivery systems are being developed to overcome this problem, including implantable devices that release medication over a prolonged period of time in, what is known in the art as, a slow release regime.

Gene transfer has gained a special importance in this context since genes can be inserted into the cells of animals or human patients where they could function, as therapeutic agents. This has led to experimenting the use of several viral vectors for introducing functional genes into cells extracted from patients, and subsequent implantation of these transfected cells for *in vivo* delivery of the desired therapeutic product[1,2,3].

The use of gene transfer, for a variety of research, medical and veterinary purposes, will be greatly facilitated if a universal transplant exhibiting long survival, and bearing no risk of malignant transformation, can be easily tailored for *in vivo* production of a desired gene product, and can be easily introduced, removed and reintroduced. Eukaryotic parasites exhibiting these features are the subject of the present invention.

Parasitism has evolved as an adaptation to life in a specialized ecological niche offered by another organism, the host, and co-evolution made parasites highly adapted to life in their host. Parasites can be found within every life form, from bacteria and plants to farm animals, laboratory animals and humans.

A spectrum of interrelations exist between parasites and hosts, ranging from a violent encounter where the parasite inflicts pathological effects and even death on its host, to a benign interaction of a non-violent mutual existence and even interdependence between host and parasite.

Such mutual existence on the genome level, as between the DNA of certain viruses and the genome of their host, has been exploited for enabling gene transfer.

For example, murine retroviral vectors, which are stably integrated into the hosts' genome, have been used in human gene therapy trials, but since they integrate with the host genome at random they can potentially induce malignancy or deleterious insertional mutations[4].

Therefore, other integrating viruses like the Adeno-associated virus vectors (AAV) are being developed for non-random integration[5]. Since integration into the host genome requires that the host cells are dividing, other systems which may be suitable for non-dividing host cell are being developed for gene transfer, adenoviruses being a notable example[6,7].

While the study of many different viruses for development of recombinant vectors for gene therapy of humans, animals and plants, is a mainstay in gene transfer/gene therapy research[1], one should consider that a long term association between host and parasite is not unique to certain viruses but also exists with much higher taxa.

Yet, the potential of unicellular or even multicellular eukaryotic parasites, for a long term production of therapeutic gene products *in vivo* has yet not been studied or exploited at all.

The potential of parasitic worms for this purpose is of particular interest because of their following advantageous features: (i) they exist in their host as separate genetic entities with independent capacity for synthesis of natural gene products, and potentially also of products of newly introduced genes; (ii) they have the qualities of universal transplants and sometime of a prolonged survival in their definitive hosts; (iii) They can be found in a variety of locations in the body where therapeutic gene products can exert their beneficial effect locally or systemically; (iv) they do not multiply as adults in their definitive host (in order to multiply their eggs have to reach the environment or intermediate hosts for further development), and

therefore it is possible to control the worm burden in the definitive host by controlling the number of the infective stages to which it is exposed, and hence to control the amount of gene products secreted by them; (v) some species of parasitic worms multiply clonally as larvae in their intermediate host, which makes possible easy propagation of transgenic forms which incorporated genes expressing desired products; (vi) elimination of the pathogenic qualities of parasitic worms is, in some cases, readily possible; (vii) elimination of the capacity of parasitic worms to continue their life cycle and cause contamination of the environment with infective forms, can be readily achieved in some cases, and in other cases can be expected pending further studies; (vii) removal of parasitic worms by effective drugs is easily possible in many cases.

All of the above features and more are shared by certain flatworms, belonging to the Genus *Schistosoma* (Bilharzia parasites), members of the Class *Trematoda* . Schistosomes are blood fukes of man and animals. Eggs of these parasites are released in the excreta and, when reaching freshwater, release the first stage larva, the miracidium, which infects certain species of freshwater snails with high host specificity. At the site of penetration the miracidium rapidly transforms to a mother sporocyst within which daughter sporocysts develop asexually. About two weeks later multiplc daughter sporocysts are released. They migrate to the snails digestive gland (hepatopancreas), and within them the cercariae, larvae infective to respective vertebrate hosts, develop asexually and are released in large

numbers to the water. An infected snail may release cercariae from about 5 weeks after infection (depending on water temperature) throughout the snail's life time (several additional weeks)[8].

As further detailed hereinunder, Schistosomes were selected as a model parasite of choice for gene transfer because of their multiple advantages. For this purpose the relevant information required for evaluating the feasibility of expressing foreign proteins by schistosomes and for developing, accordingly, the construct vectors and gene transfer conditions required for this purpose was integrated. The transgenic schistosomes were developed to serve as a model platform intended for the delivery and stable expression of a variety of desirable gene products in their intended host.

SUMMARY OF THE INVENTION

According to the present invention there are provided transgenic eukaryotic parasites for use as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals.

According to further features in preferred embodiments of the invention described below, there is provided a eukaryotic diploid multicellular parasite transduced with a transgene.

According to further features in preferred embodiments of the invention described below, there is provided a method of providing a

eukaryotic host with a protein or polypeptide comprising the step of infecting the eukaryotic host with a eukaryotic diploid parasite transduced with a polynucleotide sequence encoding the protein or polypeptide.

According to still further features in the described preferred embodiments the polynucleotide sequence is a transgene.

According to still further features in the described preferred embodiments the protein or polypeptide is secreted from the parasite.

According to still further features in the described preferred embodiments the infection is by a plurality of individuals of the parasite, all of the individuals are of a single sex.

According to still further features in the described preferred embodiments the single sex is selected from the group consisting of male and female.

According to still further features in the described preferred embodiments the parasite is a worm.

According to still further features in the described preferred embodiments the worm is a flat worm.

According to still further features in the described preferred embodiments the flat worm is a trematode.

According to still further features in the described preferred embodiments the trematode is a schistosome.

According to still further features in the described preferred embodiments the schistosome is selected from thc group consisting of

Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis.

According to still further features in the described preferred embodiments the host is human or animal and the parasite is infective to the human or animal.

According to still further features in the described preferred embodiments the parasite is sterile.

According to still further features in the described preferred embodiments the parasite is sensitive to a known drug, the drug is therefore effective in removing the parasite from the host.

According to still further features in the described preferred embodiments the polynucleotide sequence is integrated in the parasite's genome.

According to still further features in the described preferred embodiments the integration is by homologous recombination into a selected genomic locus.

According to still further features in the described preferred embodiments the selected genomic locus is a repetitive sequence.

According to still further features in the described preferred embodiments the selected genomic locus is a unique sequence.

According to still further features in the described preferred embodiments the transgene is extrachromosomal.

According to still further features in the described preferred embodiments the parasite has distinguishable sexes, whereby a single sex of the sexes is used for the infection.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a transgenic eukaryotic parasite for use as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 is a schematic representation of the glutathion-S-transferase (GST) gene from *Schistosoma mansoni*, wherein TATA indicated the location of the promoter, ex = exon, intr = intron, PstI is a site for the *Pst*I endonuclease and GST-1 and GST-2 denote the location of these respective PCT primers;

FIG. 2 is a schematic representation of a GST-GFP fusion construct, wherein TATA indicated the location of the GST promoter, ex = exon, intr = intron, the streaked PstI is a former site for the *Pst*I endonuclease which was destroyed by bluntization during the cloning procedure;

FIG 3. is a schematic representation of an GFP-Sm1-7 construct which includes the GST promoter region (TATA);

FIG. 4 is a full sequence of the GFP-GST recombinant vector including the GST-GFP fusion construct of Figure 2 (SEQ ID NO:1);

FIG. 5 is a map of the GFP-GST recombinant vector including the GST-GFP fusion construct of Figure 2;

FIG. 6 is a full sequence of the recombinant GFP-Sm1-7 vector including the GFP-Sm1-7 fusion construct of Figure 3 (SEQ ID NO:2);

FIG. 7 is a map of the GFP-Sm1-7 recombinant vector GFP-Sm1-7 fusion construct of Figure 3;

FIG. 8 shows results of PCR with cercariae, by employing GFP primers, wherein M = size markers, A = transgenic cercariae with GFP introduced inversely (control), B = transgenic cercariae with GFP introduced directly, WT = wild type cercariae and P = the GFP-GST recombinant vector of Figure 5;

FIGs. 9a-d are confocal microscopy images of cercariae with incorporated GFP-GST vector, and with control, wherein Figures 9a and 9b show GFP-positive cercariae, fluorescence (yellow-white) is seen over a red background, Figure 9c shows positive signals in transgenic cercariae

fluorescence (red) in combination with Nomarski microscopy, whereas, Figure 9d shows several wild type cercariae with negative signal (only the red background is visible);

FIGs. 10a-d are confocal microscopy images of cercariae with incorporated GFP-Sm1-7 vector (Figures 10a-c) and controls (Figure 10d);

FIGs. 11a-i are confocal microscopy of transgenic adult worms and of controls, wherein Figures 11a-d show s worm from an experiment in which GST-GFP (inverse) was employed, Figure 11e shows a wild type worm, and Figures 11f-i show a worm from an experiment where the GFP was at the direct position.

FIGs. 12a-f are confocal microscopy of transgenic adult worms and of controls, wherein Figure 12a shows a strongly positive male-female pair from a GFP-GST transgenesis, Figures 12b-c show a positive male worm from an Sm1-7 transgenesis experiment, Figures 12d-e shows two negative male-female wild type worms, whereas Figure 12f shows a female wild type worm; and

FIGs. 13a-d show confocal microscopy images obtained after staining with anti-GFP fluorescent antibodies, wherein Figures 13a-b show bodies of GFP-GST transgenic cercariae, one with Cy-5 fluorescent antibody staining (red over blue, Figure 9b), and the other with both GFP (green) and Cy-5 fluorescent antibody (red) staining (Figure 9a), and Figures 13c-d show cercariae from an experiment with GFP-SM1-7 transgenesis, stained by both GFP fluorescence and CY-5 fluorescence.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of transgenic eukaryotic parasites such as parasitic worms which can be used as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Thus, in one aspect, the present invention is of a eukaryotic diploid multicellular parasite transduced with a transgene.

As used herein in the specification and in the claims section below, the term "eukaryotic diploid" refers to organisms having a diploid set of chromosomes. It will be appreciated that, by definition, all eukaryotes are diploid in at least a part of their life cycle, typically throughout their life cycle.

As used herein in thc specification and in the claims section below, the term "multicellular" refers to organisms having differentiated cell types interacting there amongst to form a functional organism.

As used herein in the specification and in the claims section below, the term "parasite" refers to an organism which, in at least a part of its life cycle, lives on or within another specics, from which it obtains nutrients and/or shelter.

As used herein in the specification and in the claims section below, the term "transduced" also means "genetically modified" and refers to the result of a process of inserting nucleic acids into cells of a species. The insertion may, for example, be effected by transformation, injection, transfection, gene bombardment, electroporation or any other means effective in introducing nucleic acids into cells of a species, be it to all of the cells of the species or to any part or types of cells thereof, *in vivo* or *in vitro*.

As used herein in the specification and in the claims section below, the term "transgene" refers to any polynucleotide sequence transduceable into an organism or cells thereof. A transgene typically includes a polynucleotide sequence including control elements such as a promoter and enhancer sequences and a coding region encoding a polypeptide or protein, including reporter fused polypeptides or proteins. If the transgene is integrated into the genome of the parasite using homologous recombination close to control elements at the site of integration these control elements may direct the expression of the coding region of the transgene.

In another aspect the present invention is of a method of providing a eukaryotic host with a protein or polypeptide comprising the step of infecting the eukaryotic host with a eukaryotic diploid parasite transduced with a polynucleotide sequence encoding the protein or polypeptide. Since many parasites are known to have restrictive species specificity, the eukaryotic diploid parasite and the eukaryotic host are selected compatible, in other words, the parasite is selected infectious to the host.

As used herein in the specification and in the claims section below, the term "eukaryotic host" refers to a eukaryotic organism from which a parasite obtains nutrition and/or shelter.

According to a preferred embodiment of the present invention the protein or polypeptide is secreted from the parasite. To these end, the polynucleotide sequence preferably includes signal peptides for secretion. However, non-secreted proteins are also within the scope of the present invention since parasite death can cause release of the protein to the host's circulation.

The protein may be any desired protein for which a gene was isolated. Typically the protein of choice is missing, dysfunctional or ineffective in the host, whereas the parasite serves to replenish the missing, dysfunctional or ineffective protein. The protein may be a hormone, a growth factor, an enzyme, a clotting factor, a cytokine, an antigen, a receptor, a proteinaceous anti-microbial molecule, a proteinaceous neuro-transmitter, etc.

For example, the protein may be insulin for treatment of insulin dependent diabetes. The protein may be a growth hormone or sex hormone for treatment of conditions where any of these hormones is absent, dysfunctional or ineffective, or any other hormone of impaired expression in the host.

In order to control parasite burden within the host and to minimize the risk of environmental parasite pollution it is advantageous in some embodiments to infect the host with a known number of individual parasites of a single sex, either male or female, so as to avoid reproduction.

Alternatively, the parasite burden is maintained un-increased by infection with sterile (non-reproducing) parasite individuals. Sterilization may be accomplished genetically, for example by crossing heterozygotes for a recessive sterility gene, physically by removal of sex and/or reproduction organs or by irradiation or chemical treatment known to damage these organs.

Burden selection depends on various factors, including, but not limited to, the amount of gene product secreted by the parasite and the amount of gene product required by the host for reversing symptoms or treating a condition. One ordinarily skilled in the art would know how to adjust the parasite burden by monitoring the host's symptoms.

Many parasitic worms are known. These includes species belonging to certain classes of the phyla *platyhelminthes*, *Acanthocephala* and *Nematoda*. According to a preferred embodiment of the present invention the

transgenic parasite is a worm, preferably a flat worm, preferably a trematode, most preferably a schistosome, including the species *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis.* For treatment of animals, such as house hold and farm animals and animals kept in captivity a parasite compatible with that animal is selected, whereas for treatment of man, a parasite compatible with man is selected, all according to the species specificity of the parasite. One ordinarily skilled in the art would know how to select a compatible parasite species to a treated host.

According to a preferred embodiment of the present invention the parasite is sensitive to a known drug, the drug is therefore effective in removing the parasite from the host when so desired. For example, schistosomes are sensitive to a variety of schistosomicidial drugs, including, but not limited to, praziquantel, oxammquin, metrifonate, hycanthone, nicrosamide and other. However, drugs for other parasites are well known in the art.

According to a preferred embodiment of the present invention the polynucleotide sequence (transgene) is integrated in the parasite's genome. The integration may by effected by homologous recombination into a selected genomic locus, such as a repetitive sequence or a unique sequence.

Integration by homologous recombination is presently preferred since the chances of hampering genes which are important or crucial for the functionality of the parasite in general and within the host in particular are reduced. Integration into a repetitive sequence is advantageous since multiple harmless integration sites are available, increasing both the chances of integration and the number of integration events, however, these sites are disadvantageous in that in some cases they are located in non-transcribed regions of the genome. Integration by homologous recombination into unique sequences is also within the scope of the present invention. These sequences are selected according to their functionality. In order to obtain high levels of expression of the transgene, the integration site is preferably selected close to and down stream of strong and effective expression control sequences. In any case, the scope of the present invention is not limited to integration by homologous recombination, in other words non-specific or non-targeted integration is also within the scope of the present invention, such that even parasites for which no sequence information is available can be transduced. Furthermore, non integrated or extrachromosomal (e.g., episomal) transgenes are also within the broad scope of the present invention, although at present less favorable due to the possibility of diluting or loosing the transgene during cell divisions.

Due to the progress of genetic engineering techniques it is now possible to introduce any selected expressible gene to any living organism,

including parasites. Therefore, the scope of the present invention is not limited to the examples shown herein.

While conceiving the present invention and further while reducing it to practice, the concept, the procedures, the conditions and the reagents for introducing foreign genes into parasites in order to enable their use as a platform for *in vivo* production of desired gene products were thought of and practiced.

Schistosomes serve herein as a choice model system demonstrating the potential use of eukaryotic parasites in general for *in vivo* delivery of desired gene products in their host. The use of parasitic worms is presently of choice. This may include tapeworms such as *Hymenolepis diminuta* for introducing gene products into the intestines, and the filaria *Brugia malayi* for introducing gene products into the lymphatics, and into the circulation. Conditions for abrogating pathogenicity and transmission may influence parasite selection. According to the present invention transgenic schistosomes are employed where unisexual infections provide these requirements.

When foreign genes were introduced by electroporation into miracidia they were incorporated and passaged to schistosome larvae developing in the snail, and subsequently into adult worms developing in the vertebrate host.

Development of transgenic worms expressing desired transgene product (GFP in the present work) in the target recipient of this delivery system has been achieved.

The results achieved while reducing the present invention to practice demonstrate that introduction of foreign genes into schistosome miracidia within ova is applicable, and that a high proportion of transgenic cercariae was subsequently obtained after clonal multiplication in the snail host as expected.

The expected advantage of a introducing the foreign DNA by a wide scatter, e.g., by electroporation, was also confirmed. Introducing the foreign genes both into the GST gene and preferably into the SM1-7 repeated sequence of the schistosome's genome were successful although the second location seems more suitable for obtaining better cercarial and adult worm yields.

The present invention is designed to provide a modular system into which desired genes and control elements can be introduced. In the examples provided herein, the foreign genes were introduced into the schistosomal genome preferably by homologous recombination. It is demonstrated that control elements of a schistosomal gene such as GST can be employed for expressing the foreign gene, and that the foreign gene can be introduced into an established (unique) schistosomal gene or into highly repeated DNA sequences of satellite DNA. The use of alternative strong promoters which have been previously demonstrated to be active in transgenic eukaryotes, such as the SV40 promoter[19], but not excluding other promoters, can also be considered for the same purpose. Targeting of the foreign genes into genomic locations other than the GST gene and the Sm1-7 repeated sequence

can also be considered. One takes into consideration that in some cases the transfected gene may be toxic in higher concentrations, or that its expression will require external regulation. In these cases constructs with inducible promoters can be prepared. The most effective inducible promoters that are in use for transfections of eukaryotic cells are promoters with tetracycline regulatory systems[68]. Addition or depletion of tetracycline causes these promoters to start or stop transcription of the corresponding downstream gene coding regions. Other triggers of inducible promoters can be incorporated as they become available. The introduction of the construct containing the foreign gene into a satellite DNA region offers a possibility to avoid putative damage that may arise from introduction of the foreign gene within a single copy gene whose function is essential for the survival of the parasite. The introduction of foreign DNA into the schistosomal genome enable a stable expression through out its life cycle, depending on the control elements of choice. This, however, does not exclude introduction of the desired genes as episomes including the desired gene in a recombinant vector. It also does not exclude introduction of the desired foreign genes into other locations such as mitochondria[69], intra-cytoplasmic locations, and artificial chromosomes[70, 71], as well as into other possible locations not mentioned here. The high efficiency of introducing foreign genes into the miracidium while in the ovum is demonstrated herein below. Electroporation proved suitable for introducing foreign genes into schistosomes, but biolistics, should be suitable for this purposes too as can be expected from previous work with eukaryotic

cells[15]. Other methods such as lipofection and chemical transfection , but not excluding additional methods, are also possible now that the principles and feasibility of producing transgenic schistosomes have been demonstrated. Introduction of the foreign DNA into the miracidium while it is enclosed within the ovum was selected for this work but this does not exclude introducing the DNA into other stages. The non-motile mother sporocyst, which can be obtained *in vitro* directly by transformation from miracidia[72] is a very likely stage into which foreign genes can be introduced and which can then be transplanted directly into the snail host as is done with daughter sporocysts taken from infected snails[29]. Since schistosomes multiply clonally within their intermediate host (the snail) introduction into miracidia or sporocysts and then infection of the snail (naturally for miracidium or artificially for the sporocyst) should enable expansion of transgenic clones. Infection of the snail by a single miracidium or sporocyst will ensure a single-sex progeny of cercariae for a long period of shedding (weeks). Once the transgenic nature of the cercariae as well as their sex[59] are determined by PCR with appropriate sex chromosome specific primers, and gene products identified by an adjoining reporter gene, and or by specific detection of transgene products, the clone can be expanded to any desired size by transplantation of the transformed sporocysts into naive snails[29]. In this way "farming" of desired transgenic clones can be accomplished and cercariae thereof can be taken "from the shelf" at any time for introduction into a few or into many target animals or humans at the same time.

Abrogating the parasites' pathological responses is a crucial requirement for employing parasites for delivery of desired gene products. It is therefore important to analyze carefully the dynamics of schistosome infection and disease. The best known model for this purpose is *S. mansoni* infection in laboratory rodents and the following summary is a generally accepted overview[73]. *S. mansoni* worms develop to maturity (and egg laying capacity) within five weeks. Eggs in the tissues require one week to mature and then survive for three weeks. During the first five weeks of infection Th1 cells are dominant in the murine immune response with IFN-g and IL-2 as dominant lymphokines. After egg laying begins, Th2-type responses become dominant with IL-4, IL-5 and IL-10 as dominant lymphokines. IL-4 secretion is associated with IgE response and IL-5 with eosinophilia. IL-10 down regulates the Th1 responses. At about 12 weeks of infection immunological down-regulation occurs, accompanied by down regulation of the granulomatous response around *S. mansoni* eggs and by a reduced collagen synthesis.

At the early stages of infection[8], acute symptoms of fever, diarrhea, abdominal pain, myalgia, arthalgia, emaciation, cough, hepatomegaly and urticaria were the most frequent symptoms reported [74, 75]. Immune complexes appear to have an important role in the acute disease, and its resemblance to serum sickness (resulting from the well established therapeutic use of antitoxines) has been noted[76, 77].

In the baboon, which has been proposed as a model for acute schistosomiasis mansoni, the onset of symptoms coincides with the onset of egg laying[47]. In the mouse too, early lesions induced by mature eggs appears as the major factors in the pathogenesis of acute schistosomiasis mansoni[46].

In humans, on the other hand, in a few cases of those reported with a known time of onset of infection, symptoms of acute disease were said to have started even before egg laying[74, 75]. These findings, although limited, raise the question of whether transgenic unisexual schistosomes may in some cases cause acute disease symptoms in humans (as opposed to animals).

Consideration of this possibility is therefore required when initial trials of the new technology in humans are carried out. The limited information so far available[74,75] is not clear-cut about the exact time of exposure to infection in humans, because the time of exposure was determined based on questionnaires and epidemiological information. Also, the possibility that the symptoms were caused by other, coinciding, causative agents was not excluded, and finally, even if pre-oviposition symptoms of acute schistosomiasis did occur in humans, it is not possible under natural conditions to separately characterize them (in terms of nature, severity and duration) from post-oviposition symptoms. Only actual infections of humans selected for treatment by unisexual transgenic parasites will make it possible to clarify the net pathogenicity of worms. It is also important to mention in this context that egg antigens and antibodies to egg antigens passed from

mothers to fetuses were suggested to protect against acute disease[78]. This finding reinforces the presumed role of the eggs in the pathogenesis of acute disease in humans too, and it also suggests that a suitable immune manipulation may abrogate acute disease (if eventually found in controlled unisexual human infections).

The present invention is suitable for *in vivo* release by the parasites of a variety of desired products of transgenes. It will enable preparation of parasites carrying more than one transgene, or introduction of more than one population of transgenic schistosomes into target hosts for multiple effects, when necessary.

It will primarily enable introduction of transgenic larvae (cercariae or schistosomula), which will complete development in the recipient within several weeks. But transplantation of fully developed worms grown in animals for immediate effects will also be possible, but will be logistically more difficult. Among the gene products that can be delivered by the present invention are the following, not excluding others not mentioned, hormones and growth factors, enzymes, clotting factors, cytokines, antigens, receptors, anti-microbial molecules, neuro-transmitters, etc. These can be used in three main biomedical/animal sciences domains as follows.

For preparing laboratory animals presenting features for basic scientific research purposes: Over- expression of desired gene products; expression of inhibitors of natural gene-products; correction (total or graded) of the phenotypic outcomes of knocking out genes through breeding or

genetic engineering; altered susceptibility to disease conditions that may be caused by agents such as, but not limited to, pathogens, chemicals, pharmaceuticals, natural products, environmental factors, etc.; altered fecundity and embryonic development; altered behavior; etc. Since *S. mansoni* which was used in this work readily infects mice, this embodiment of the present invention is suitable for immediate application.

For veterinary purposes: Over-expression of desired gene products; expression of inhibitors of natural gene products; correction (total or graded) of genetic or acquired deficiencies; altering the rate of development and of gaining body mass; altering fecundity including sperm count and oestral cycle; altering efficiency of milk production; altering the rate of production of transgene products in transgenic farm animals (e.g., in milk) and in clones thereof; altering susceptibility to disease agents such as microbes, but not excluding other agents; etc.

For medical purposes: Restoration of deficiencies whether genetic[1] or acquired, such as, but not limited to, hormonal deficiencies (for example, deficiencies in growth hormone and insulin), metabolic deficiencies (for example, deficiency in metabolic enzymes), hematological deficiencies (for example, deficiencies in clotting factors), immunological deficiencies (for example, adenosine deaminase (ADA) deficiency), etc.; immunotherapy (including vaccination requiring long term antigenic stimulation, and over production of cytokines); anti-microbial therapy (including the anti-viral action of interferon, and microbe-binding soluble receptors for eliminating

disease agents such as HIV); anti-cancer therapy; treatment of drug addiction; treatment of a variety of poisoning conditions; amelioration of geriatric conditions; etc. Since *S. mansoni* is a parasite of man this embodiment of the present invention is suitable for immediate clinical practice.

The procedure proposed in accordance with the teachings of the present invention for prospective uses in animal sciences, should enable obtaining phenotypic features of the target animals, which otherwise could be achieved only by the complex, time consuming and much more expensive breeding or genetic engineering/transgenesis for each single property in each host strain.

It should make treatment of humans with *in vivo* transgenes, universally compatible and therefore much more readily available and inexpensive.

In addition, the procedure disclosed avoids genotypic alteration of patients, thus avoiding the accompanying risks of mutagenesis and malignant transformation.

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

EXAMPLE 1

Theoretical and Experimental Considerations

This preliminary part of the work involved assembly of data into an integrated theoretical concept for preparing transgenic schistosomes intended for *in vivo* delivery of desired gene products in their host's circulation. This part of the work led to the formulation of relevant working hypotheses, and to relevant experimental designs. In the core of this integrated concept are the putative advantages of schistosomes for the proposed purposes, as follows:

Introduction of foreign genes into schistosomes should be very easy, and much safer than direct gene transfer to humans: Schistosomes are separate genetic entities within their host. Thus making them transgenic for expressing desired genes within the host, should be much safer than introducing foreign genes directly into the host's genome, a manipulation which may result in malignant transformation or insertional mutations harmful to the host[1]. Gene transfer into worms has so far been successful only in the free living non-parasitic nematode *Caenorhabditis elegans*[9]. The success in this case is due to the simple and rapid (a few days) life cycle of this organism. Thus, injection of recombinant expression vectors into the oocytes within the ovarium resulted in a proportion of transgenic larvae which developed into fast-multiplying transgenic adults. This approach is less likely to succeed in schistosomes because the *in-vitro* culture of adults with subsequent egg laying and maturation of an infective miracidium of this obligate endo-parasite is complex[10], and the life cycle of schistosomes is

long (requiring about 3 months to be completed in the snail and vertebrate hosts). Since vigorous asexual (clonal) multiplication of schistosome larvae occurs within the snail[8], it would be best to achieve a high proportion of transgenesis of parasites just before they start to multiply within the snail so that transgenic organisms will be amplified during intramolluscan multiplication (see below). Miracidia, schistosome larvae which infect snails by active penetration, can therefore be considered the prime target for transgenesis. The miracidium is a ciliated multicellular organism with 4 epidermal plates arranged in 4 tiers and covered with cilia and apical musculature and glandular structure suitable for penetrating the snail host. Most of the posterior 1/3 part of the organism is filled by a cluster of germinal cells which are interconnected and are also connected to the surface of the organism[11]. Germinal cells undergo multiplication shortly after the miracidium penetrates into the snail and transforms into a mother sporocyst[8,11], which is the start of asexual multiplication to form daughter sporocysts and subsequently cercariae[8,11]. In addition, miracidia are highly acidiophilic[11] and are located in highly perforated ova[12] capable of allowing passage of macromolecules[13]. These features combined (in particular the connection between germ cells among themselves and with the surface of the organism, as well as their acidophilicity[11]), seem theoretically favorable for introducing DNA into miracidia. Hitting miracidia with a wide scatter of DNA is a likely approach for successful introduction of DNA into their germ cells. Targeting miracidia while they are within eggs seems a more

reasonable approach since the highly motile free miracidia in water may escape an effective hit or may be relatively more vulnerable. Introduction of the foreign DNA may be achieved by electroporation[14], or by biolystics[15]. Chemical transfection[16] by $(Ca)_2(PO_4)_3$, $CaCl_2$ or the like can also be considered if miracidia will not be adversely affected by these chemicals, and lipofection[17] (by using micelles or liposomes containing DNA) may also be suitable. As further detailed below, it was chosen to transfect schistosome miracidia within their eggs by electroporation, and to use the putative transgenic miracidia for infecting snails, and then to establish that transgenic cercariae and subsequently transgenic adult worms expressing products of the transgene, can be recovered.

There is a choice of genomic locations suitable for introducing foreign genes into schistosomes: Schistosomes have a diploid genome with 16 chromosomes (2n=16)[18]. The control of gene expression in schistosomes has not been sufficiently defined and a system for introducing foreign DNA into the parasite has not yet been developed. For expression and secretion of desired gene products in schistosomes it is important that control elements responsible for high degree of expression and secretion will operate in the environment of the schistosomal cell. Many of the control elements generally employed are species specific for the target organism of trasnsfection, so that elements working properly in a mammalian system may not be suitable for lower organisms like schistosomes. Many expression vectors utilize promoter and other control elements derived from viral

genomes. For instance, LTRs from the promoter of retroviruses or SV40 can, when active, enable high levels of gene expression[19]. These may indeed be suitable for gene transfer in schistosomes too. A more specific approach is to combine the foreign gene with control elements of the target organism (schistosomes in our case) and introduce the construct into the selected location in the target genome by homologous recombination[20,21]. Thus, the expression of the foreign gene which was integrated into the target genome will be under the control of the target gene into which it was integrated. Expression will therefore be stable and the newly introduced gene will be replicated stably with the recipient genome. Several schistosomal genes have been cloned and characterized, and all the genes which express a protein which is secreted, such as Glutathion-S-transferase[22, 23], Sm 31/32[24] and others[25, 26, 27], seem suitable for our purposes. With more information accumulating on schistosomal genes, more candidates for integrating foreign genes into the schistosomal genome are likely to emerge. An alternative site for introducing foreign genes into schistosomes is an abundant repeated sequence of 121 bp units tandemly arranged which was found in the genome of *S. mansoni*[28]. When introducing foreign genes into schistosomes the selection of transgenic organisms by selective markers seems less suitable than by reporter genes because selection may be hampered by the slow life cycle and the multicellular nature of the organism. Suitable reporter genes may actually enable identification of transgenic sporocysts in a dissected snail for transfer into naive snail for propagation and maintenance[29] (see

below). Various reporter genes like the genes for chloramphenicol acetyl transferase (*CAT*)[30], b galactosidase (*b-Gal*)[31], luciferase (*luc*)[32], or green fluorescent protein of jellyfish (GFP)[33, 34]. The latter, although less sensitive than *luc* by 3 orders of magnitude, has recently been widely used in transgenesis because its expression in a transgenic animal indicates that expression is efficient far beyond the detection limit of the expression products. GFP was hence selected as a reporter genc in this work.

Schistosomes multiply clonally in their intermediate host, and this is expected to greatly facilitate expansion and "farming" of desired transgenic clones: Schistosomes multiply clonally in freshwater snails in a way that presents an important advantage for propagation of transgenic larvae. As already mentioned the miracidium transforms at the penetration site into a mother sporocyte within which germ balls are formed which develop into daughter sporocytes. Daughter sporocysts then emerge and migrate to the snails digestive gland, the hepatopancreas, and germ-balls within them continuously develop into thousands of cercariae (the stages infective to man or other hosts) which continue to be shed from the snail throughout his life, generally for many weeks[8,11]. Since all subsequent larvae developing from a single miracidium are a clone, starting snail infection by exposing it to a single miracidium will result in a unisexual infection. As will be subsequently explained (see below), a unisexual infection is required to prevent the major, cgg related pathology. Snails shedding cercariae of the desired sex can be selected by examining cercariae

by the polymerase chain reaction (PCR) with sex specific primers[35]. Furthermore, long term maintenance of desired clones can be done by serial transfer of sporocysts (within each sporocyst a clone of cercariae is continuously produced) from snail to snail by implantation[29], thus potentially enabling maintenance of "a farm" of transgenic clones. Each clone in such a "farm" will release transgenic cercariae carrying a certain transgene, and each can be expanded as desired by implantation into additional naive snails. As already mentioned miracidia within the eggs were selected for the present work as the target of transfection.

The anatomical location of schistosomes, their size, and excretion of products by them are expected to be of advantage for employing them as a transgenic platform for expressing and delivering desired genes in vivo: Schistosomes reside in the mesenteric-portal veins or in the vesical plexus (depending on the species). They are capable of secreting macromolecules[36] which should be accessible into the blood, with subsequent systemic distribution. With regard to their residence in veins they are unique among parasitic worms, although access of materials secreted by parasitic worms to the hosts' vasculature also occurs among nematodes causing lymphatic filariasis which reside in the lymphatics[37]. Adult schistosomes may occasionally be found in ectopic locations[38], and may be theoretically transplanted in blood vessels of additional organs where the substances they secrete may be required for therapy. In adult schistosomes ongoing turnover of the outer membrane regularly releases membrane constituents into the

blood stream[39]. Other constituents are secreted as has been described above[22-27]. These findings which demonstrate that schistosomes can release gene products into the circulation are strengthened by actual demonstration of circulating schistosomal constituents in the circulation of the host[24]. Schistosomes are about 1 cm long, but the biomass of the male is several times larger than that of the slender female, and its maturation is independent of the presence of the female. By contrast, the slender female requires continuous contact with the male in order to complete its growth and maintain sexual maturity. The female is permanently carried by the male in a special "gynecophoric" canal and studies have shown the passage of nutrients and developmental stimuli from male to female[40,41,42]. These differential features make the schistosome male a preferred candidate for *in vivo* production of desired gene products.

The pathogenicity of schistosomes can be ready abrogated as required for their use as universal transplants: In vertebrates schistosome pathogenicity depends mainly on their capacity to lay eggs, a proportion of which do not succeed to be passed out with excreta, but become trapped in the host's tissues (of the intestines and liver or of the bladder-depending on the schistosome species). Antigens secreted from the trapped eggs induce a vigorous immune granulomatous response and subsequent fibrosis[43]. These T-dependent immune responses[44] lead to the structural and hemodynamic changes typical of the disease (schistosomiasis or Bilharzia)[8]. At conditions where eggs are not produced, like in the case of unisexual infection, even

large number of worms do not cause the typical granulomatous disease, as was found in mice, the common model of egg-induced pathology in experimental schistosomiasis mansoni[45,46]. In schistosomes, where eggs cause most of the pathology, unisexual, "eggless", infection can be achieved very easily since all the cercariae shed by a snail which was infected by a single miracidium are unisexual[45] and sterilization for preventing egg laying is unnecessary. The early stage of infection by *S. mansoni* in humans has been known to cause an acute disease[8]. Whether or not eggs are an important factors in causing acute disease in humans, as is the case in murine schistosomiasis mansoni[46] and in *S. mansoni*-infected baboons[47], will be discussed. Eggs deposited in the intestines and the liver of animals and humans, infected with *S. mansoni* induce pathology in the intestines and in the liver. In chronic hepatosplenic schistosomiasis mansoni porto-systemic anastomoses may develop due to portal hypertension which develops as a result of the cumulative egg-related hepatic pathology. In such cases eggs and occasionally worms can be found in the lungs and to a lesser extent a variety of ectopic anatomic locations. The pathology in most such cases is caused by the eggs[8] but even ectopic adult schistosomes were not surrounded by inflammatory cells[38], except perhaps rarely when they die in the lungs[48]. The pathologic effects of dying worm are probably minimal as evident from the fact that although killing of adult *S. mansoni* worms by treatment with schistosomicidal drugs has been accomplished in millions of people (many with a high worm burden), symptoms associated with dying worms have

generally not been recorded. Only in a few cases worm death was associated with acute disease[8].

Schistosomes have a long life span suitable for a prolonged expression of transgenic gene products in vivo: *S. mansoni* can live for decades as it was found in Yemeni immigrants to Israel[49]. Their prolonged survival in the definitive host points to the existence of mechanisms of immune evasion[50]. Even when an immune response develops against these parasites it will affect the young worms (schistosomula) at various stages after penetration and migration within the definitive host, and even then, mechanisms of immune attrition can affect only a fraction of the re-infecting larvae[50]. In man, immunity against schistosomes can be detected during puberty after a prolonged infection[51,52], although partial age-dependent resistance may also exists[52,53]. Various humoral factors have been demonstrated to participate in human immunity to schistosomes[54] while the role of cellular mechanisms is as yet unclear. Also, various antigens have been proposed as protective antigens and are being studied as candidate antigens for vaccination[55]. The role of the eggs on the development of immunity and resistance against *S. mansoni* (the most studied schistosome species) is well documented in animal models[56,57], therefore infection by unisexual transgenic schistosomes will most likely not only avoid the major pathological feature of schistosomiasis as described above, but may also decrease attrition of the parasites and thus enable even longer survival than recorded for natural infection of man[52]. Schistosomes can therefore be

considered as well adapted, long living universal transplants in their specific hosts, and transgenic schistosomes can be expected too to survive for a long time in their host.

Schistosomes do not multiply in their vertebrate host and they can be easily introduced into their hosts or removed from it, to enable control of the worm burden and hence of the quantity of transgene products: Cercariae of schistosomes penetrate actively through the skin of target host when it becomes exposed to waterborne cercariae by dipping[8,11]. Cercariae can alternatively be injected into the skin directly or following artificial transformation to schistosomula, which can even be cryopreserved for long term storage[58]. Following penetration cercariae transform rapidly into schistosomula which migrate via the blood stream to the lungs and then to the liver where they mature, and subsequently migrate to their target location. Since the sex of the cercaria can be easily determined by the polymerase chain reaction (PCR)[59] the identification and selection of unisexual males, the transgenic transplant of choice, can be readily done. It was already mentioned that the worm burden can be determined by the size of the infective dose, and that replenishing is possible until a desired therapeutic effect is achieved. In addition, removal of schistosomes is possible by a number of new and effective drugs (notably praziquantel) which are being widely used for individual and community-based chemotherapy. Prazyquantel is very effective by a single oral dose (sometimes divided) and yields a high percentage of cure[60]. This should potentially enable removal

of transgenic worms from treated individuals at any desired time. The monitoring of schistosome worm burden is possibly by examining circulating antigens in their host[61]. This should make possible control of schistosome worm burden.

Prevention of environmental dissemination of transgenic schistosomes can be expected to be straightforward: The capacity of schistosomes to be transmitted in nature depends on presence of specific infected snails shedding cercariae in sites where definitive hosts (humans or animals) contact the water. Egg-bearing excreta are the source of snail infection and therefore introducing unisexual transgenic cercariae into target hosts is not expected to result in environmental contamination with eggs.

In addition to schistosome species infecting man there are those which infect farm animals, thus the new technology should be suitable for animal experimentation for basic science purposes as well as for veterinary/animal husbandry purposes: The hosts of various schistosome species occurring in Africa are well known[62]. Among them *S. mansoni* and *S. haematobium* are essentially human parasites, but other species of African schistosomes can infect cattle, sheep, goat, and other ruminants of economic importance. In addition, *S. japonicum* which infects people in the Far East also infects 31 species of wild mammals and 13 species of domestic animals including dog, pig, cow, water buffalo and goat[8]. All species of schistosomes can be used for the proposed technology. GST genes of schistosomes other than *S. mansoni*[63] have been identified[64], and may be

used as optional sites for inserting desired genes. Also, highly repeated sequences similar to the one described to *S. mansoni*[28] are also present in the genome of other schistosome species including *Schistosoma haematobium* (see SEQ ID NO: 9, disclosing a *DraI* repeat sequence in the genome of *Schistosoma haematobium*, estimated to present in 50,000 copies per genome), and are potential sites of gene insertion. The new technology presented here for *in vivo* expression of desired gene product by the parasite, can expand the use of the mouse, and of other laboratory animals compatible for schistosome infection, for a variety of basic and applicable research purposes.

EXAMPLE 2

Materials and Methods

Parasite strain, maintenance of its life cycle and harvesting of parasites: *Schistosoma mansoni*, an Egyptian strain, was originally obtained from Wellcome Laboratories 35 years ago and its life cycle maintained by standard methods in outbred albino mice and in its snail host *Biomphalaria glabrata*. Briefly, livers were collected from infected mice about 9 weeks after subcutaneous injection with 350 cercariae. Numerous ova surrounded by granulomata were embedded in the liver tissue. The livers were homogenized and the eggs washed several times in 1.7 % NaCl solution (to avoid miracidial hatching under inadvertent hypotonic conditions). Distilled water was added to the final egg sediment and hatching of miracidia was

enabled for 30 minutes. Miracidia were collected from the egg suspension in a dark room and under a narrow beam of light (which attracts these phototrophic larvae). Snails were exposed in separate small containers to 5-10 miracidia each for 16 hours under fluorescent light source. At least 24 snails were infected as a batch. Six or more weeks later, after completion of asexual multiplication of larvae within the snails, cercariae were shed from the snails under light in a beaker containing dechlorinated water, and 7 week old female mice were injected subcutaneoulsy with 350 cercariae. *S. mansoni* clean eggs for electroporation were harvested as previously described[65,66] from intestines of mice infected 9 weeks previously with wild type (WT) schistosomes. Adult worms were isolated from the mice 6-10 weeks later by liver perfusion[67].

Selection of electroporation conditions: Clean schistosome eggs were sedimented by centrifugation (1000 rpm/3 min) and washed once in 10 ml of cold RPMI1640 medium supplemented with geneticin and with 15 % fetal calf serum. Finally the eggs were resuspended in RPMI, and a 0.2 ml egg suspension (containing 2.5×10^3 or 5×10^3 eggs) transferred to an electroporation cuvette (0.4 cm wide) and exposed to various eletroporation conditions as follows in a Gene Pulser (Bio-Rad) with exponential discharge: 500 V, 750 V, 100 V and 1500 V from a 25 μF capacitor; 1500 V and 2000 V from a 0.25 μF capacitor; 250 V and 450 V from a 125 μF capacitor and from a 500 μF capacitor. Electroporated eggs were left at room temperature for 20 minutes then 0.8 ml double distilled water was added for hypotonicity

and the quvettes left for 0.5 hours under light, for enabling hatching of miracidia. Snails were exposed individually to 5-10 miracidia/snail for 16 hours under light. Hatching and viability (motility), were subsequently determined in search of electroporation conditions that will yield about 50 % viability of miracidia. For determining hatching rate, the content of each cuvette was transferred to a well of a 24 well culture plate, lugol was added for killing the miracidia and for staining, and about 100 ova and miracidia in the same area were screened. The number of free miracidia and of empty ova (eggshells) were determined and the average between them provided the hatching rate (% hatching). For determining viability by motility, the contents of each cuvette was centrifuged at 1000 rpm for 2 minutes to sediment eggs and dead miracidia and motile miracidia in the supernatant were collected, immobilized and stained with lugol, and counted. Control counts were carried out with eggs kept under similar conditions but without pulsing.

Based on the results obtained from preliminary experiments (see Results below) the following conditions were employed in the present work: Eggs (0.2 ml suspension containing $2.5x10^3$ or $5x10^3$ eggs) were mixed with plasmid DNA (5-10 mg/$5x10^3$ eggs) then transferred to an electroporation cuvette (0.4 cm wide) and exposed to the selected electroporation conditions. These were exponential discharge of 500 V (1250 V/cm) from a 25 μF capacitor. Electroporated eggs were left at room temperature for 20 minutes

then 0.8 ml double distilled water was added for hypotonicity and the quvettes left for 0.5 hours under light, for enabling hatching of miracidia.

Following the development of the parasites after gene pulsing: Snails were exposed individually to 5-10 miracidia/snail for 16 hours under light. Cercariae were shed beginning from 6 weeks later as described above (see section on maintenance of life cycle). Development of cercariae was followed by periodic (weekly to triweekly) determination of numbers cercariae shed from snails. In initial experiments only approximation was employed by grading cercarial shedding from "a few cercariae" (+) through "very strong shedding" (++++). These grades were given numerical values as follows: 10 (+), 100 (++), 500 (+++) and 1500 (++++). In subsequent experiments numbers of cercariae/snail were determined. Cercariae harvested from snails infected with transduced miracidia underwent analysis confocal microscopy (see below) or were employed to infect mice, which in turn were a source of adult worms, collected 7-10 weeks later by perfusion[67].

Isolation of cercarial genomic DNA: Cercariae were shed from infected snails infected by wild type (WT) or electroporated miracidia from the sixth week after exposure to miracidia. Genomic DNA was isolated from cercaria by lysing cells in 0.1 % Triton x 100 + TEN (10 mM Tris-HCl pH 8.0, 10 mM EDTA and 10 mM NaCl). The lysate was treated with RNase A (0.1 mg/ml) in 37 ^{o}C for 30 minutes and then with Proteinase K (0.1 mg/ml) in 37 ^{o}C for 30 minutes. Residual proteins were removed from the lysate by

phenol extraction followed by phenol-chloroform extraction. Genomic DNA was recovered by ethanol precipitation.

Analysis of genomic DNA: Aliquots of *Schistosoma mansoni* cercarial DNA (>1 ng) underwent amplification by the polymerase chain reaction (PCR) with primers specific for the GFP gene at an internal position (see paragraph on primer design and PCR conditions, below). Products of the PCR were separated by agarose gel electrophoresis and stained with ethidium bromide.

Plasmids construction: All the plasmids employed were derived from the commercially available pBluescript plasmids (Stratagene). Into the *Eco*RV restriction enzyme site of these plasmids introduced was a 1900 bp long fragment from the 5' end of genomic copy of the GST gene[23] isolated from genomic DNA of *Schistosoma mansoni* by PCR reaction using specific primers GST-1 and GST-2 (see paragraph on primers design and PCR conditions, below). Figure 1 provides a schematic representation of the GST gene.

The GFP gene which encodes the green fluorescence protein from the medusa *Aequorea victoria*[33,34] was than inserted into the PstI site located in the second exon of the GST gene[23] ("GST-GFP fusion gene"). The GFP coding sequence that was used, was obtained from plasmid pGFP by PCR reaction with specific primers covering most of the entire gene (see paragraph on primer design, below). The size of the GFP fragment was designed to fit the reading frame of the GST protein in exon 2. The resulting

construct contains a fusion of the GST-GFP proteins in the same reading frame. This construct is expected to produce the fluorescent protein under the regulation of gene expression of GST from *Schistosoma*. This fusion protein also contains the peptide sequences that are targeting this protein to cellular compartments were the native GST protein resides, namely passage through the tegument of the parasite. Due to the long homology between the plasmid harboring the GFP and the GST genomic sequences, the cassette of GFP-GST can be incorporated by homologous recombination and stably expressed as part of the genome. Alternatively, GFP protein with the GST leader and under regulation of the GST promoter sequence may be transiently expressed by the vector positioned as an episome. The GFP was also introduced inversely for obtaining a control construct in which expression is not expected. Figure 2 provides a schematic representation of the GST-GFP fusion gene.

In order to achieve better incorporation of the expression vectors into the Schistosomal genome the long homology of the GST gene was replaced with shorter homology of the sequence SM1-7. This sequence is found in the genome in high copy numbers (10 %)[28] therefore the vector may target many sites in the genome. The regulatory sequences that were used in this type of vector included the promoter of GST. For construction of this vector the major part of the 5' end of the GST sequence was deleted by digestion with *Cla*1I and *Afl*III restriction enzymes, as well as all of the 3' end of this gene in the GST-GFP fusion vector, leaving only thc promoter region (the first 200

nucleotides of the GST gene). Five copies of SM1-7 sequence were introduced into the *XhoI* and *XbaI* restriction enzyme sites flanking the GST promoter of the GFP gene fusion. Figure 3 provides a schematic representation of this construct.

Primer design and PCR conditions: Two sets of primers were used in plasmids construction, the GST primers were used for amplification of the GST gene from the genome of *Schistosoma* and the second set of primers was used to amplify the GFP coding region from plasmid pGFP. The computer program Oligo4 was used to select the optimal and unique primers for both genes.

Primers to amplify the GST gene were :

GST-1: 5'- CATCGAGAACGGTTTACATGTTCA-3' (SEQ ID NO:3)

GST-2 : 5'- GCAGCCTCCTCACATGCTCCA-3' (SEQ ID NO:4)

The PCR for amplification of the GST gene was performed under the following conditions: 30 cycles of: denaturation - 94 °C, 60 sec; annealing - 55 °C, 60 sec; and elongation - 72 °C, 3 min.

Primers to amplify the GFP gene were :

uGFP: 5'- ATGAGTAAAGGAGAAGAACTTTTC-3' (SEQ ID NO:5)

lGFP: 5'- TTTGTATAGTTCATCCATGCC-3' (SEQ ID NO:6)

A pair of internal primers specific for the GFP gene were designed for detection of GFP in transfected Cercariae by PCR. These were as follows:

GFP1: 5'- TCTCCCATGATGTATACATTATGT-3' (SEQ ID NO:7)

GFP2: 5'- TCTCCATCGAAGGGTCATCACG-3' (SEQ ID NO:8)

The PCR for amplification of the GFP gene and fragments was performed under the following conditions: 25 cycles of: denaturation - 94 ^{o}C, 30 sec; annealing - 51 ^{o}C., 30 sec; and elongation - 72 ^{o}C 60 sec.

Sequencing: The sequences of the constructs developed were confirmed by double stranded sequencing carried out automatically by the Dye Deoxy Sequencer (Applied Biosystems). The sequencing results are shown in Figures 4 and 6 (SEQ ID NOs: 1 and 2, respectively).

Examination of cercariae and adult worms by confocal microscopy: Cercariae shed from snails infected with WT miracidia or with miracidia that underwent electroporation with recombinant plasmids were analyzed directly after they were shed, or following fixation with 3 % paraformaldehyde, treatment with 50 mM NH_4Cl, and washing with PBS. Adult worms were collected from infected mice and examined directly or following fixation. Scanning laser confocal microscope (Zeiss 410) was employed to examine cercariae and adults by laser excitation at 488 nm, employing an excitation filter FT 510 and an emission filter LT 515. Contrast level used was 310-320.

Examination of cercariae and adult worms by anti-GFP: This examination was to confirm the presence of GFP in the transfected parasites. The indirect fluorescent antibody technique (IFAT) was employed for this purpose with anti-GFP monoclonal antibody (Clontech, USA) as a first antibody, and Cy5-labeled goat anti-mouse IgG (Chemicon, USA), as a second antibody. Parasites were incubated at room temperature for 0.5 hours

(cercariae) or one hour (adult worms) with 3 % formaldehyde in PBS, then washed once with PBS and incubated for 0.5 hours at room temperature in 50 mM NH_4Cl for blocking free aldehyde groups, and washed again with PBS. Permeabilization was carried out by incubation at room temperature for 0.5 hours with a solution containing 0.1 % triton X-100 and 1 % BSA in PBS, then washing with PBS. Blocking was carried out by incubation for 0.5 hours at room temperature in 5 % Goat serum. The first antibody (diluted 1:500) was reacted with the parasites at 37 °C for 0.5 hours or overnight at 4 °C and following four washes in PBS the second antibody (diluted 1:400) was reacted with the parasites for 0.5 hours at 37 °C. The parasites were examined by confocal microscopy after mounting in a solution containing 86 % glycerol, 10 % PBS, 0.1 % NaN_3, and 3-4 % DABCO (1,4 Diaza Bicyclo (2.2.2) Octane. The scanning laser confocal microscope (Zeiss 410) was employed with excitation by a Helium-Neon Laser at 650 nm, and emission at 680 nm.

EXAMPLE 3

Experimental Results

Sequence of our recombinant plasmids: Figure 4 presents the sequence of the vector carrying the GFP-GST fusion coding information (SEQ ID NO:1). Figure 5 presents the map of this rccombinant plasmid. Figure 6 presents the sequence of the vector in which the GFP was inserted

within the SM1-7 repeated sequence (SEQ ID NO:2). Figure 4 presents the map of this recombinant plasmid

Electroporation conditions: Percent hatching of miracidia from ova which underwent electroporation was higher than in control unpulsed ova in 6 out of 10 electroporation conditions tested. Percent hatching was similar to control in 2 electroporation conditions (25 µF/1500 V and 0.25 µF/ 2000V), and lower than control values in 2 other electroporation conditions (25 µF/ 500V, and 0.25 µF/1500 V). The results of one experiment (out of two) are presented in the Table 1 below.

TABLE 1

PULSE:	0	25 µF----------------------------- >				0.25 µF-------->		125 µF------->		500 µF------->	
		500 V	750 V	1000 V	1500 V	1500 V	2000 V	250 V	450 V	250 V	500 V
% HATCH:	20	18.8	28	23.4	20.5	14.4	20.2	29.7	25.4	29.4	26.6

Percent motility of miracidia hatched from ova which underwent electroporation was invariably lower than that obtained with control unpulsed ova. The results of one experiment are presented in Table 2 below.

TABLE 2

PULSE:	0	25 µF------------------------------->				0.25 µF--------->		125 µF-------->		500µF-------->	
		500 V	750 V	1000 V	1500 V	1500 V	2000 V	250 V	450 V	250 V	500V
% MOTILE:	46	20*	8	7	2	45	40	34	23**	12	8

* Selected conditions; **Immediate alternative option.

In three subsequent experiments where cercarial viability (motility) was measured, the selected electroporation conditions (25 µF/500 V) were

employed for transducing foreign DNA (5-10 mg/cuvette). The count of motile miracidia was quarter to half of the control.

Rate of cercariae development: Cercariae were shed in a pool of snails (at least 10 snails/pool but usually more at the time of shedding). Cercarial shedding in normal schistosome/snail combination typically starts 4 weeks after exposure to 5-10 miracidia, it usually reaches a maximal rate ranging between 1000 and 2800 cercariae/snail, and the peaks of cercarial shedding is between days 60 and 90 following exposure. In two control experiments where electroporation was done without the presence of DNA, cercarial shedding reached normal high values, but the peak was somewhat delayed (between 80 and 110 days following exposure) and remained high until the last measurement taken (124 days following exposure). In 4 out of 8 transduction experiments with the GFP-GST fused construct, cercarial counts were very low with "tens" of cercariae/snail or less recovered throughout the monitoring period (up to 200 days after the snails were exposed to miracidia). In two out of these 8 experiments very strong shedding started only 120 days, or 160 days after exposure to miracidia. In another two out of these 8 experiment cercarial shedding peaked early (at about 60 days after exposure) and reached over 2000 cercariae/snail for 2-3 weeks, then declined rapidly. In one of these two later experiments electroporation conditions were milder (25 μF/400V instead of 25 μF/500 V). In 2 out of 4 experiments in which the GFP-SM1-7 construct was employed for transformation, cercarial shedding was with multiple peaks reaching several hundred cercariae/snail (range of

500 to 800). In one of these 4 experiment a single low peak (about 200 cercariae/snail) was exhibited at about 100 days post exposure. In one of these 4 experiments altered electroporation conditions were employed (25 μ F/400 V instead of 25 μF/500 V). High infection was obtained from the very early cercarial shedding (42 days) and fluctuated between 1200 cercaria/snail and about 2700 cercariae/snail. Table 3 below summarizes the results of cercarial counts.

TABLE 3

Miracidia used to infect snails	Maximum No. cercariae/snail	Time of high cercariae no./snail (> 1000)	Duration of high shedding
Wild type	1-2.5 $\times 10^3$	day 60 to 90 PE[a]	60 days
Pulsed W/O DNA	+ + + +	day 80-130 (estimate)	50 days (estimate)
Pulsing control	+ + + +	day 80-130 (estimate)	50 days (estimate)
"GFP in GST"			
Exp. # 3:	+ + + +	day 164 to 193 PE[a]	Min. 26 days
Exp. # 5:	+ + + +	day 123 to 142 PE[a]	Min[b]. 21 days
Exp. # 6:	+ + (one + + +)	Practically irrelevant	
Exp. # 7:	+ + (one + + +)	Practically irrelevant	
Exp. # 8:	+ +	Practically irrelevant	
Exp. # 9:	+ +	Practically irrelevant	
Exp. # 15:	2500	day 47-118 PE[a]	Min[b]. 71 days
Exp. # 16[c]:	2000	day 49-106 PE[a]	Min[b]. 54 days
"GFP in Sm1-7"			
Exp. # 13:	+ + + (under 1000)	day 55 to 146 PEa	
Exp. # 14:	+ +	Practically irrelevant	
Exp. # 15:	+ + (a single + + +)	Practically irrelevant	
Exp. # 16[b]:	2750	day 43 to 106 PEa	Min[b]. 73 days

Relative values for cercarial shedding: marginal (+), weak (+ +), medium (+ + +), strong(+ + + +).
a = Post Exposure; b = Minimum. c = reduced voltage (see text).

It can be concluded for practical purposes that introducing foreign genes may cause a reduction in the number of shed cercariae or a delay in cercarial shedding. The trend with cercariae to which GFP-SM1-7 construct was introduced, was towards a more efficient shedding. It therefore ensues

that ongoing monitoring of cercarial shedding is required with several replicate experiments, and a clone exhibiting desired qualities may then be selected for propagation by transplantation of sporocysts to normal snails and for infecting the vertebrate host.

Rate of development of adult worms: In two experiments where mice were exposed to cercariae from experimental snails to which the GST-GFP construct was introduced as made evident by PCR (70-75 cercariae/mouse for active skin penetration) no adult worms were recovered. When wild type cercariae are used for the active penetration method, the number of adult worms recovered is about 25 % of the number of cercariae to which mice were exposed. In other experiments, cercariae were injected subcutaneously. When wild type cercariae are used for infecting mice by injection, the number of adult worms recovered fluctuates between 1 % and 10 % of the number of cercariae introduced. The results of these experiments with transgenic cercariae were as summarized in table 4 below.

TABLE 4

Identity of cercariae injected	No. of recovered adult worms/mouse
GST-GFP transgenic	3, 0, 30, 3, 21
GST-GFP (inverse) transgenic	27, 10, 0
Sm-7-GFP transgenic	5, 20, 18, 17, 33, 42, 52
Wild Type	5, 30, 5, 25

The trend with cercariae transduced with the GFP-SM1-7 construct was towards a more efficient development into adults. Also, in 3 out of the 7 mice in this group all adult worms were males.

Incorporation of GFP gene into the schistosomal genome: Figure 8 presents a representative PCR experiment demonstrating amplification of the GFP region by corresponding primers, when PCR was carried out with total genomic DNA prepared from cercariae. Amplification signals were demonstrated whether the GSP sequence was introduced directly (B), or inversely (A) within the schistosomal genome. Introduction of the transgenes into the schistosomal genome was thus demonstrated. However, simultaneous presence of the recombinant vector as an episome was not excluded. Demonstration by PCR of the incorporation of the GFP-SM1-7 transgene into the schistosomal genome is still required (although expression was clearly demonstrated by confocal microscopy as described below).

Expression of GFP in transgenic schistosomes:

a. Cercariae: GFP was expressed by most of the cercariae examined (1-10/group depending on availability) in some experiments only part of the cercariae (20-60 %) were positive. Although a statistical analysis was not done due to the small number of cercariae examined in each experimental group, these results suggest a very efficient transgenesis. When more than one miracidium is used for infecting a snail a mixture of positive and negative cercariae is not surprising considering that not all of the miracidia became effectively transfected. Positive signals were diffuse or concentrated in discrete foci. Representative results of confocal microscopy with cercariae are presented in Figures 9a-d and 10a-d. Figures 9a-d demonstrates a gallery of 4 photographs of experiments where the GFP-GST construct was

employed. GFP-positive cercariae are demonstrated in Figures 9a-c, whereas Figure 9d is of a group of wild type cercariae. In Figures 9a-b fluorescence (yellow-white) is seen over a red background. In Figure 9c fluorescence (red) is seen in combination with Nomarski microscopy. Figure 9d shows several cercariae with negative signal (only the red background is visible). Figures 10a-d present a gallery of photographs, three (Figures 10a-c) with positive signals from experiments where the GFP-Sm1-7 construct was introduced, and one (Figure 10d) wild type as control. Please note the pronounced fluorescence observable in the experimental images (Figures 10a-c) as compared with control (Figure 10d).

*b. **Adult worms:*** Figures 11a-i present a gallery of nine photographs as follows: Figures 11a-d show a worm from an experiment in which the GST-GFP (inverse) was employed, Figure 11e shows a wild type worm, and Figures 11f-i show a worm from an experiment in which the GST-GFP was employed. Strong expression typified the male worm in Figures 11f-i. Focal fluorescence (autofluorescence) at the tips (mouth and tail) of the male worm of Figures 11a-d was also observed. Focal fluorescence at the anterior was also present in the wild type worm of Figure 11e. Figures 12a-f present a gallery of six photographs of adult worms, in which Figure 12a shows a strongly positive male-female pair from a GFP-GST transgenesis, Figures 12a-b show two positive male worms from an GFP-Sm1-7 transgenesis experiment, Figures 12d-e show two negative male-female wild type worms, and Figure 12f show a negative female wild type worm showing non specific

fluorescence of the ovarium (non specific fluorescence of the gut is also found when pigment resulting from digestion of hemoglobin is present).

Anti-GFP tests: Figures 13a-d present a gallery of four photographs taken with transgenic cercariae which underwent fluorescent antibody staining. The photographs of Figures 13a-b were taken with bodies of GFP-GST transgenic cercariae, one of them (13b) with fluorescent Cy-5 labeled antibody staining (red over blue) staining, and the other with both GFP (green) and Cy-5 fluorescent antibody (red) staining. The other 2 cercariae shown in Figures 13c-d were from an experiment with GFP-SM1-7 transgenesis. They were viewed by both GFP and CY-5 staining. Differences in intensity can be observed. Examining adult worms is particularly important because of the non specific fluorescence observed in some cases at the anterior and posterior ends and at the position of the ovarium.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Joseph Hamburger and Avraham Laban

(ii) TITLE OF INVENTION: TRANSGENIC EUKARYOTIC PARASITES AS UNIVERSAL GRAFTS FOR USE IN VIVO DELIVERY OF BENEFICIAL GENE PRODUCTS IN HUMANS AND IN ANIMALS

(iii) NUMBER OF SEQUENCES: 8

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 1.44 megabyte, 3.5" microdisk

(B) COMPUTER: Twinhead® Slimnote-890TX

(C) OPERATING SYSTEM: MS DOS version 6.2, Windows version 3.11

(D) SOFTWARE: Word for Windows version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILLING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILLING DATE:

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(B) TELEFAX: 972-3-5625554

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5356

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6494

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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AACGAAATTT GTATTGCTTT GCTCCAGCTT TTGTTCCCTT TAGTGAGGGT 3650
TAATTGCGCG CTTGGCGTAA TCATGGTCAT AGCTGTTTCC TGTGTGAAAT 3700
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CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG 4000
GATAACGCAG GAAAGAACAT GTGAGCAAAA GGCCAGCAAA AGGCCAGGAA 4050
CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG 4100
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GCTGGCGAAA GGGGGATGTG CTGCAAGGCG ATTAAGTTGG GTAACGCCAG 6400
GGTTTTCCCA GTCACGACGT TGTAAAACGA CGGCCAGTGA GCGCGCGTAA 6450
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATCGAGAAC GGTTTACATG TTCA 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCAGCCTCCTC ACATGCTCCA 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGAGTAAAG GAGAAGAACT TTTC 24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTTGTATAGT TCATCCATGC C 21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTCCCATGA TGTATACATT ATGT 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCTCCATCGA AGGGTCATCAC G 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 121

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCTCACCT ATCAGACGAA ACAAAGAAAA TTTTAAAATT GTTGGTGGAA 50
GTGCCTGTTT CGCAATATCT CCGGAATGGT TGGTCGTATC GTTGTGAAAA 100
TTGTTTCATA TTATTGGTGA C 121

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WHAT IS CLAIMED IS:

1. A eukaryotic diploid multicellular parasite transduced with a transgene.

2. The parasite of claim 1, wherein the parasite is a worm.

3. The parasite of claim 2, wherein said worm is a flat worm.

4. The parasite of claim 3, wherein said flat worm is a trematode.

5. The parasite of claim 4, wherein said trematode is a schistosome.

6. The parasite of claim 1, wherein the parasite is infective to human or animal.

7. The parasite of claim 1, wherein the parasite is infective to human.

8. The parasite of claim 5, wherein said schistosome is infective to human.

9. The parasite of claim 5, wherein said schistosome is selected from the group consisting of *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis.*

10. The parasite of claim 1, wherein the parasite is sterile.

11. The parasite of claim 1, wherein the parasite is sensitive to a known drug.

12. The parasite of claim 1, wherein said transgene is integrated in the parasite genome.

13. The parasite of claim 1, wherein said transgene is integrated in the parasite genome.

14. The parasite of claim 1, wherein said transgene is integrated in a selected genomic locus in the parasite genome.

15. The parasite of claim 14, wherein said selected genomic locus is a repetitive sequence.

16. The parasite of claim 14, wherein said selected genomic locus is a unique sequence.

17. The parasite of claim 1, wherein said transgene is extrachromosomal.

18. The parasite of claim 1, wherein the parasite has distinguishable sexes.

19. A method of providing a eukaryotic host with a protein or polypeptide comprising the step of infecting the eukaryotic host with a eukaryotic diploid parasite transduced with a polynucleotide sequence encoding the protein or polypeptide.

20. The method of claim 19, wherein said protein or polypeptide is secreted from said parasite.

21. The method of claim 19, wherein said infection is by a plurality of individuals of said parasite, all of said individuals are of a single sex.

22. The method of claim 21, wherein said single sex is selected from the group consisting of male and female.

23. The method of claim 19, wherein said parasite is a worm.

24. The method of claim 23, wherein said worm is a flat worm.

25. The method of claim 24, wherein said flat worm is a trematode.

26. The method of claim 25, wherein said trematode is a schistosome.

27. The method of claim 19, wherein said host is human or animal and said parasite is infective to said human or animal.

28. The method of claim 19, wherein said host is human and said parasite is infective to human.

29. The method of claim 26, wherein said schistosome is infective to human.

30. The method of claim 26, wherein said schistosome is selected from the group consisting of *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis*.

31. The method of claim 19, wherein said parasite is sterile.

32. The method of claim 19, wherein said parasite is sensitive to a known drug, said drug is therefore effective in removing said parasite from said host.

33. The method of claim 19, wherein said polynucleotide sequence is integrated in said parasite's genome.

34. The method of claim 33, wherein said integration is by homologous recombination into a selected genomic locus.

35. The method of claim 34, wherein said selected genomic locus is a repetitive sequence.

36. The method of claim 34, wherein said selected genomic locus is a unique sequence.

37. The method of claim 19, wherein said transgene is extrachromosomal.

38. The method of claim 19, wherein said parasite has distinguishable sexes, whereby a single sex of said sexes is used for said infection.

ABSTRACT OF THE DISCLOSURE

A eukaryotic diploid multicellular parasite transduced with a transgene. A method of providing a eukaryotic host with a protein or polypeptide including the step of infecting the eukaryotic host with a eukaryotic diploid parasite transduced with a polynucleotide sequence encoding the protein or polypeptide.

Attorney Docket. 325/8
page 1 of 2

Combined Declaration For Patent Application and Power of Attorney

As a below named Inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled TRANSGENIC EUKARYOTIC PARASITES AS UNIVERSAL GRAFTS FOR USE IN VIVO DELIVERY OF BENEFICIAL GENE PRODUCTS IN HUMANS AND IN ANIMALS.
the specification of which

(check one) ☒ is attached hereto

☐ was filed on as Application Serial No. and was amended on . I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
NONE (number)	(Country)	(Day, Month, Year Filed)	☐ Yes	☐ No
(number)	(Country)	(Day, Month, Year Filed)	☐ Yes	☐ No
(number)	(Country)	(Day, Month, Year Filed)	☐ Yes	☐ No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

NONE (Application Serial No.)	(Filing Date)	Status (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	Status (patented, pending, abandoned)

I hereby appoint the following attorneys, with full power of substitution, association, and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Mark M. Friedman Registration No. 33,883

Address all Correspondence to:

DR MARK FRIEDMAN LTD. c/o ANTHONY CASTORINA 2001 JEFFERSON DAVIS HIGHWAY SUITE 207 ARLINGTON, VIRGINIA 22202	Direct all telephone calls & faxes to: ROBERT SHEINBEIN Phone (703) 415-1581 Fax (703) 415-4864

Attorney Docket: 325/8
page 2 of 2

Continuation of Combined Declaration For Patent Application and Power of Attorney

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application of any patent issued thereon.

*FULL NAME OF SOLE OR FIRST INVENTOR	INVENTOR'S SIGNATURE	DATE
JOSEPH HAMBURGER	J. Hamburger	24 nov. 1998
RESIDENCE HASNUNIT	CITIZENSHIP	
10/3 ~~SNUNIT~~ ST. MEVASERET ZION, ISRAEL	ISRAELI	
POST OFFICE ADDRESS		
10/3 SNUNIT ST. MEVASERET ZION, ISRAEL		

*FULL NAME OF SECOND INVENTOR	INVENTOR'S SIGNATURE	DATE
AVRAHAM LABAN		29. Nov. 1998
RESIDENCE	CITIZENSHIP	
163 HAKFIR ST. JERUSALEM, ISRAEL	ISRAELI	
POST OFFICE ADDRESS		
163 HAKFIR ST. JERUSALEM, ISRAEL		

*FULL NAME OF THIRD INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
	ISRAELI	
POST OFFICE ADDRESS		

*FULL NAME OF FOURTH INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
	ISRAELI	
POST OFFICE ADDRESS		

*FULL NAME OF FIFTH INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
	ISRAELI	
POST OFFICE ADDRESS		

*FULL NAME OF SIXTH INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
	ISRAELI	
POST OFFICE ADDRESS		

SMALL BUSINESS CONCERN - NEW APPLICATION

Attorney Docket No.: 325/8

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In RE Application of: JOSEPH HAMBURGER ET AL.

Filed Concurrently Herewith

For: TRANSGENIC EUKARYOTIC PARASITES AS UNIVERSAL GRAFTS FOR USE IN VIVO DELIVERY OF BENEFICIAL GENE PRODUCTS IN HUMANS AND IN ANIMALS

VERIFIED STATEMENT UNDER 37 CFR 1.27
CLAIMING STATUS AS A SMALL ENTITY

To The Commissioner of Patents and Trademarks:

I hereby declare that:

I am the owner of, or an official empowered to act on behalf of, the small business concern identified below:

Name of Concern: YISSUM RESEARCH AND DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM

Address : 46 JABOTINSKI, 91042 JERUSALEM, ISRAEL

The small business concern identified above, together with its affiliates, employs fewer than 500 persons and qualifies as a small business concern as defined in 37 CFR 1.9(d) for purposes of paying reduced fees under 35 USC § 41(a) and § 41(b) to the Patent and Trademark Office with regard to the above-entitled invention described in the specification filed herewith.

Rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above entitled invention.

If the rights held by the small business concern are not exclusive, each other party having rights to the invention is listed below, and no rights to the invention are held by any party who could not qualify as a small entity under 37 CFR 1.9(f), namely any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Full Name (Party 1) : NONE

Address :

Status : ☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Full Name (Party 2) :

Address :

Status : ☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I acknowledge the duty under 37 CFR 1.28(b) to file, in this application, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the issue fee due after the date on which status as a small entity is no longer appropriate.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application and any patent issuing thereon.

Nurit Inbar	N. Inbar	November 25, 1998
Name of Person Signing	Signature	Date

Capacity of Person Signing: Secretary of the Company

Address of Person Signing : 46 Jabotinsky St. POB 4279
Jerusalem 91042, Israel

YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM

Fig 1

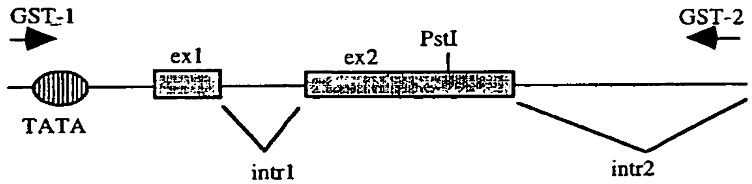

Fig. 2

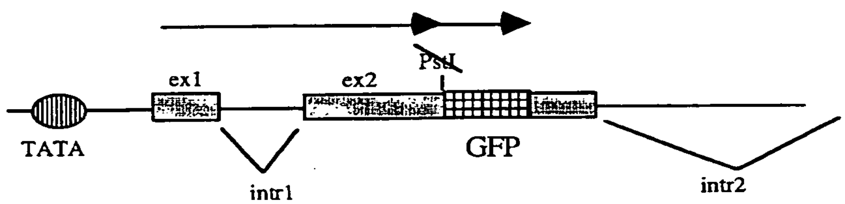

Fig. 3

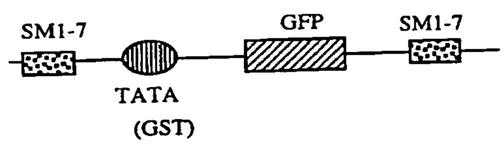

Figure 4

GSTGFP Length: 5356 February 18, 1998 09:24 Type: N Check: 5900 ..

features:

GFP gene: 56-769
the first part of the genomic GST gene: 4460-5350
the second part of the genomic GST gene: 769-1600
the Amp resistance gene (complementary): 3642-2809

```
  1 AGCTTGCATG CCTGCAGGTC GACTCTAGAG GATCCCCGGG TACCGGTAGA
 51 AAAAATGAGT AAAGGAGAAG AACTTTTCAC TGGAGTTGTC CCAATTCTTG
101 TTGAATTAGA TGGTGATGTT AATGGGCACA AATTTTCTGT CAGTGGAGAG
151 GGTGAAGGTG ATGCAACATA CGGAAAACTT ACCCTTAAAT TTATTTGCAC
201 TACTGGAAAA CTACCTGTTC CATGGCCAAC ACTTGTCACT ACTTTCTCTT
251 ATGGTGTTCA ATGCTTTTCA AGATACCCAG ATCATATGAA ACGGCATGAC
301 TTTTTCAAGA GTGCCATGCC CGAAGGTTAT GTACAGGAAA GAACTATATT
351 TTTCAAAGAT GACGGGAACT ACAAGACACG TGCTGAAGTC AAGTTTGAAG
401 GTGATACCCT TGTTAATAGA ATCGAGTTAA AAGGTATTGA TTTTAAAGAA
451 GATGGAAAGA TTCTTGGACA CAAATTGGAA TACAACTATA ACTCACACAA
501 TGTATACATC ATGGCAGACA AACAAAAGAA TGGAATCAAA GTTAACTTCA
551 AAATTAGACA CAACATTGAA GATGGAAGCG TTCAACTAGC AGACCATTAT
601 CAACAAAATA CTCCAATTGG CGATGGCCCT GTCCTTTTAC CAGACAACCA
651 TTACCTGTCC ACACAATCTG CCCTTTCGAA AGATCCCAAC GAAAAGAGAG
701 ACCACATGGT CCTTCTTGAG TTTGTAACAG CTGCTGGGAT TACACATGGC
751 ATGGATGAAC TATACAAATA GCATTCGTAG AATTCTATTG CACGGTATAT
801 GGCGAAGAAA CATCATATGA TGGGTGAAAC AGACGAGGAA TACTATAGTG
851 TTGAAAAGTT GATTGGTCAG GTGAGTTGAG CCTTTTATTG GTTGATGGGA
```

Fig. 4 (continued)

901 TTTTATGCTA CTGGAGTCCT GTAAGTTCAG TTTGTTGCGT TGAATAAATA

951 AAGTTCGAAT TGATAGGCGA GTGGTAACCT ATGTGTTGTG ATTACTAAAA

1001 GTCATAAGTG TGAGGTATCC CAATTATTAG AAATGTGACT AAGACTGAGC

1051 TTTTCTGCCT ATTGGGATAT TACAGATAAG TTACTTATTT GTAGTATCGT

1101 AATAGTGTTC ACTGGGAACC ATCTAATTCA CTCTCAAAAA TACTTTATCG

1151 TGTCTTATTT TATTCATCCT CATAACTCGT TTGCTAGGAG AACAGCTGTG

1201 CCCATAGGTT AGTTGACAAG TTACTTACTT CCCCATGATA CGCCTTATTT

1251 TAATAACCTA ATAAAGGCTC GGTTACGTAA CGTCCCTATA GTGCATAGCA

1301 TACCTACGAA CATTGACTCC CAACTGATTA AGTGCGGATT GCATTTTTTT

1351 GGATGTTATC GCACAGTAAG ACAATACCAT CCTCATCTCA ATGTCAAGAG

1401 GTCTTTCTCC AGGCAACAGA TCCATACCAC CATTACTTAC ATCCATCAGA

1451 GCTGTTCCCA GAATGTCTTG TTATAAACGG ATGGATTATT TATTTATTTG

1501 ACCACATAAA TATTGCATCA AAGAGGTGGG GGATCCACTA GTTCTAGAGC

1551 GGCCGCCACC GCGGTGGAGC TCCAGCTTTT GTTCCCTTTA GTGAGGGTTA

1601 ATTGCGCGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG

1651 TTATCCGCTC ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA

1701 AAGCCTGGGG TGCCTAATGA GTGAGCTAAC TCACATTAAT TGCGTTGCGC

1751 TCACTGCCCG CTTTCCAGTC GGGAAACCTG TCGTGCCAGC TGCATTAATG

1801 AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCTCTTCCG

1851 CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG

1901 GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA

1951 TAACGCAGGA AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC

2001 GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC

Fig. 4 continued

2051 GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCGACAGGA

2101 CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC

2151 TGTTCCGACC CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG

2201 GAAGCGTGGC GCTTTCTCAT AGCTCACGCT GTAGGTATCT CAGTTCGGTG

2251 TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG CACGAACCCCC CGTTCAGCCC

2301 GACCGCTGCG CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG

2351 ACACGACTTA TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG

2401 CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC

2451 GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT

2501 TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG

2551 CTGGTAGCGG TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA

2601 AAAGGATCTC AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA

2651 GTGGAACGAA AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA

2701 GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC

2751 TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG

2801 TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT

2851 GACTCCCCGT CGTGTAGATA CTACGATACG GGAGGGCTTA CCATCTGGCC

2901 CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC TCCAGATTTA

2951 TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC

3001 AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG

3051 TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC CATTGCTACA

3101 GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG

3151 TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG

Fig. 4 (continued)

3201	CGGTTAGCTC	CTTCGGTCCT	CCGATCGTTG	TCAGAAGTAA	GTTGGCCGCA
3251	GTGTTATCAC	TCATGGTTAT	GGCAGCACTG	CATAATTCTC	TTACTGTCAT
3301	GCCATCCGTA	AGATGCTTTT	CTGTGACTGG	TGAGTACTCA	ACCAAGTCAT
3351	TCTGAGAATA	GTGTATGCGG	CGACCGAGTT	GCTCTTGCCC	GGCGTCAATA
3401	CGGGATAATA	CCGCGCCACA	TAGCAGAACT	TTAAAAGTGC	TCATCATTGG
3451	AAAACGTTCT	TCGGGGCGAA	AACTCTCAAG	GATCTTACCG	CTGTTGAGAT
3501	CCAGTTCGAT	GTAACCCACT	CGTGCACCCA	ACTGATCTTC	AGCATCTTTT
3551	ACTTTCACCA	GCGTTTCTGG	GTGAGCAAAA	ACAGGAAGGC	AAAATGCCGC
3601	AAAAAAGGGA	ATAAGGGCGA	CACGGAAATG	TTGAATACTC	ATACTCTTCC
3651	TTTTTCAATA	TTATTGAAGC	ATTTATCAGG	GTTATTGTCT	CATGAGCGGA
3701	TACATATTTG	AATGTATTTA	GAAAAATAAA	CAAATAGGGG	TTCCGCGCAC
3751	ATTTCCCCGA	AAAGTGCCAC	CTGACGCGCC	CTGTAGCGGC	GCATTAAGCG
3801	CGGCGGGTGT	GGTGGTTACG	CGCAGCGTGA	CCGCTACACT	TGCCAGCGCC
3851	CTAGCGCCCG	CTCCTTTCGC	TTTCTTCCCT	TCCTTTCTCG	CCACGTTCGC
3901	CGGCTTTCCC	CGTCAAGCTC	TAAATCGGGG	GCTCCCTTTA	GGGTTCCGAT
3951	TTAGTGCTTT	ACGGCACCTC	GACCCCAAAA	AACTTGATTA	GGGTGATGGT
4001	TCACGTAGTG	GGCCATCGCC	CTGATAGACG	GTTTTTCGCC	CTTTGACGTT
4051	GGAGTCCACG	TTCTTTAATA	GTGGACTCTT	GTTCCAAACT	GGAACAACAC
4101	TCAACCCTAT	CTCGGTCTAT	TCTTTTGATT	TATAAGGGAT	TTTGCCGATT
4151	TCGGCCTATT	GGTTAAAAAA	TGAGCTGATT	TAACAAAAAT	TTAACGCGAA
4201	TTTTAACAAA	ATATTAACGC	TTACAATTTC	CATTCGCCAT	TCAGGCTGCG
4251	CAACTGTTGG	GAAGGGCGAT	CGGTGCGGGC	CTCTTCGCTA	TTACGCCAGC
4301	TGGCGAAAGG	GGGATGTGCT	GCAAGGCGAT	TAAGTTGGGT	AACGCCAGGG

Fig. 4 (continued)

4351 TTTTCCCAGT CACGACGTTG TAAAACGACG GCCAGTGAGC GCGCGTAATA

4401 CGACTCACTA TAGGGCGAAT TGGGTACCGG GCCCCCCCTC GAGGTCGACG

4451 GTATCGATAA GCTTGATTCA TCGAGAACGG TTTACATGTT CAATGAATCG

4501 AGTCAAATTT GTCTGCTTAA TTTTTATTGG TCACTCTTTC ACAGCCAATG

4551 AGGCACTCAA TAAACAGCGA ATAGAAATGA AATATTTACA GTTAAAATCA

4601 AGAGTTACAC TATTGGCCGA TCTGTTTACT AAATGACTAT TTAATAGTCA

4651 CAGCTCAGAT AGTTAGCACT GTTTTGTCTA TTTGCAAGAT GGCTGGCGAG

4701 CATATCAAGG TGGGTTGGTG TGGTTGTTCG TCATTGTGTT GCTTGGTAAA

4751 TTTTCGAGTG TTGTTAAGGT TCCTAATTGC AGCATTTAGC GGAATAAATA

4801 TTATTCCGCT ATATGAAGGT AAATACTGAG TCCATGTTAA TTGCACATTA

4851 GATCCGTACA TATATTTGGC TGTGATTTTC CACGTCATCA GGTCAGTAAG

4901 TTACGTGTTC ACACTGGGTT CTGATATAAT GCACAACCAT TATAGTCACA

4951 TAGTGGTCAC CGATTATGTG TTAGGTGTAG CATTTCAGGC GATGTTATAG

5001 TTTGTCTGTG GAATTGTTAC GAGAACTTAG GGGATTCTAG AGCGTGAATC

5051 CATCTAGTCC ATATCTCAGA GACATGCGTC CATGGAACAG TTATTTATTA

5101 TGTGATGTTT TGTCTGAAGT GGTTTTTTTC AAGCGTAAAT TGATGTGAAA

5151 CACAGCTGTA ATTTCTATAG GTTATCTATT TTGACGGACG CGGACGTGCT

5201 GAATCGATTC GGATGACTCT TGTGGCAGCT GGTGTAGACT ACGAAGATGA

5251 GAGAATTAGT TTCCAAGATT GGCCAAAAAT CAAACCAACT ATTCCAGGCG

5301 GACGATTGCC TGCAGTGAAA GTCACTGATG ATCATGGGCA CGTGAAATGG

5351 ATGTTA

Fig. 5

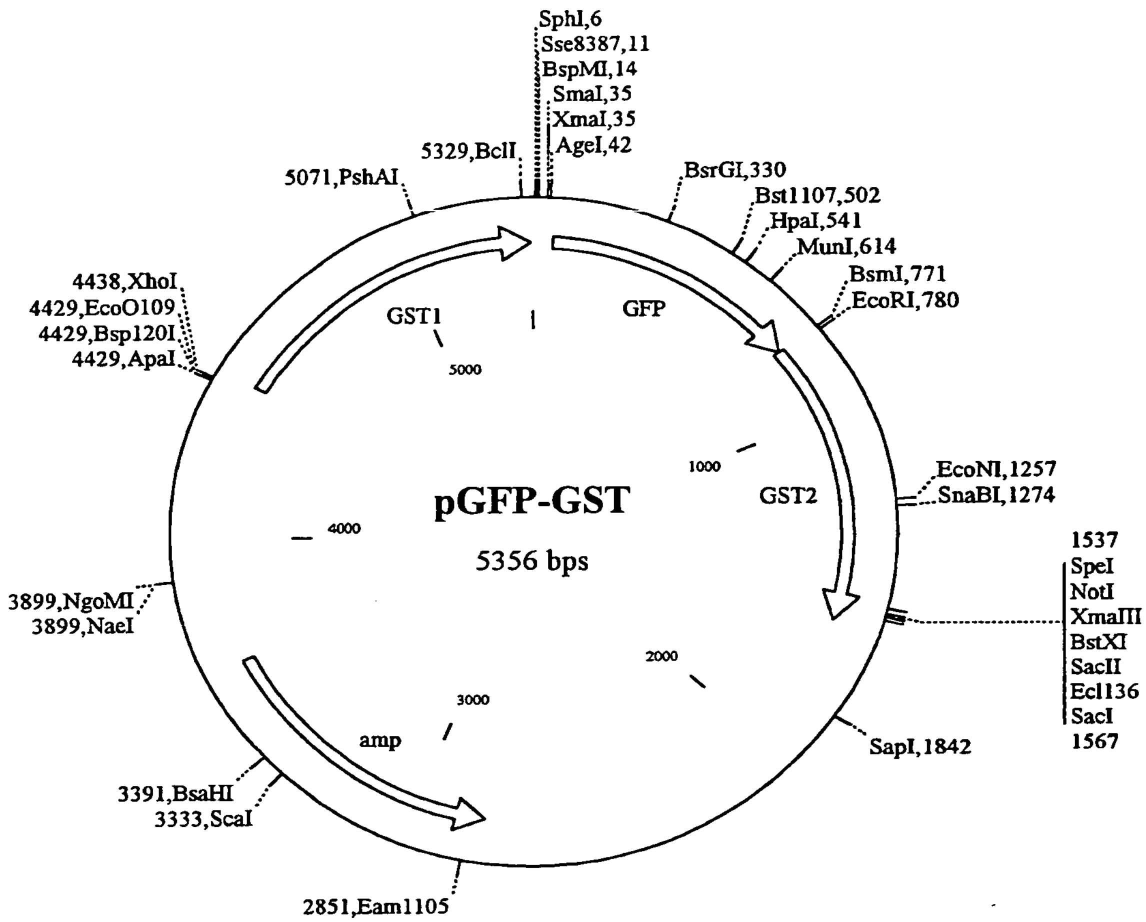
pGFP-GST
5356 bps
SphI,6
Sse8387,11
BspMI,14
SmaI,35
XmaI,35
AgeI,42
BsrGI,330
Bst1107,502
HpaI,541
MunI,614
BsmI,771
EcoRI,780
EcoNI,1257
SnaBI,1274
1537
SpeI
NotI
XmaIII
BstXI
SacII
Ecl136
SacI
1567
SapI,1842
2851,Eam1105
3333,ScaI
3391,BsaHI
3899,NaeI
3899,NgoMI
4429,ApaI
4429,Bsp120I
4429,EcoO109
4438,XhoI
5071,PshAI
5329,BclI
GST1
GFP
GST2
amp
1000
2000
3000
4000
5000

Figure 6

pSm1-7 GFP SEQUENCE

Length: 6494 February 26, 1998 11:50 Type: N Check: 8163

Features:

first sm repeat	0-560
GST1 gene	560-1520
GFP gene	1530-2265
GST2 gene	2270-3022
second sm repeat	3025-3585

```
   1  GATCTGAATC CGACCAACCG TTCTATGAAA ATCGTTGTAT CTCCGAAACC
  51  ACTGGACGGA TTTTTATGAT GTTTGTTTTA GATTATTTGC GAGAGCGTGG
 101  GCGTTAATAT AAAACAAGAA TGATCTGAAT CCGACCAACC GTTCTATGAA
 151  AATCGTTGTA TCTCCGAAAC CACTGGACGG ATTTTTATGA TGTTTGTTTT
 201  AGATTATTTG CGAGAGCGTG GGCGTTAATA TAAAACAAGA ATGATCTGAA
 251  TCCGACCAAC CGTTCTATGA AAATCGTTGT ATCTCCGAAA CCACTGGACG
 301  GATTTTTATG ATGTTTGTTT TAGATTATTT GCGAGAGCGT GGGCGTTAAT
 351  ATAAAACAAG AATCATCTCA ATCCCATCAG CCGTTCTATG AAAATCGTTG
 401  TATCTCCGAA ACCACTGGAC GGATTTTTAT GATGTTTGTT TTAGATTATT
 451  TGCGAGAGCG TGGGCGTTAA TATAAAACAA GAATGATCTG AACACGGGTG
 501  TTTTTCTGTT CAGCTTATGC AACTTTAAAA TTCGATGGGT CGTCTCAACG
 551  AAATTTGTAT TGCTTTGTCG AGGTCGACGG TATCGATAAG CTTGATTCAT
 601  CGAGAACGGT TTACATGTTC AATGAATCGA GTCAAATTTG TCTGCTTAAT
 651  TTTTATTGGT CACTCTTTCA CAGCCAATGA GGCACTCAAT AAACAGCGAA
 701  TAGAAATGAA ATATTTACAG TTAAAATCAA GAGTTACACT ATTGGCCGAT
 751  CTGTTTACTA AATGACTATT TAATAGTCAC AGCTCAGATA GTTAGCACTG
 801  TTTTGTCTAT TTGCAAGATG GCTGGCGAGC ATATCAAGGT GGGTTGGTGT
 851  GGTTGTTCGT CATTGTGTTG CTTGGTAAAT TTTCGAGTGT TGTTAAGGTT
 901  CCTAATTGCA GCATTTAGCG GAATAAATAT TATTCCGCTA TATGAAGGTA
 951  AATACTGAGT CCATGTTAAT TGCACATTAG ATCCGTACAT ATATTTGGCT
1001  GTGATTTTCC ACGTCATCAG GTCAGTAAGT TACGTGTTCA CACTGGGTTC
1051  TGATATAATG CACAACCATT ATAGTCACAT AGTGGTCACC GATTATGTGT
1101  TAGGTGTAGC ATTTCAGGCG ATGTTATAGT TTGTCTGTGG AATTGTTACG
1151  AGAACTTAGG GGATTCTAGA GCGTGAATCC ATCTAGTCCA TATCTCAGAG
1201  ACATGCGTCC ATGGAACAGT TATTTATTAT GTGATGTTTT GTCTGAAGTG
1251  GTTTTTTTCA AGCGTAAATT GATGTGAAAC ACAGCTGTAA TTTCTATAGG
1301  TTATCTATTT TGACGGACGC GGACGTGCTG AATCGATTCG GATGACTCTT
```

Fig. 6 (continued)

1351 GTGGCAGCTG GTGTAGACTA CGAAGATGAG AGAATTAGTT TCCAAGATTG

1401 GCCAAAAATC AAACCAACTA TTCCAGGCGG ACGATTGCCT GCAGTGAAAG

1451 TCACTGATGA TCATGGGCAC GTGAAATGGA TGTTAAGCTT GCATGCCTGC

1501 AGGTCGACTC TAGAGGATCC CCGGGTACCG GTAGAAAAAA TGAGTAAAGG

1551 AGAAGAACTT TTCACTGGAG TTGTCCCAAT TCTTGTTGAA TTAGATGGTG

1601 ATGTTAATGG GCACAAATTT TCTGTCAGTG GAGAGGGTGA AGGTGATGCA

1651 ACATACGGAA AACTTACCCT TAAATTTATT TGCACTACTG GAAAACTACC

1701 TGTTCCATGG CCAACACTTG TCACTACTTT CTCTTATGGT GTTCAATGCT

1751 TTTCAAGATA CCCAGATCAT ATGAAACGGC ATGACTTTTT CAAGAGTGCC

1801 ATGCCCGAAG GTTATGTACA GGAAAGAACT ATATTTTTCA AAGATGACGG

1851 GAACTACAAG ACACGTGCTG AAGTCAAGTT TGAAGGTGAT ACCCTTGTTA

1901 ATAGAATCGA GTTAAAAGGT ATTGATTTTA AAGAAGATGG AAACATTCTT

1951 GGACACAAAT TGGAATACAA CTATAACTCA CACAATGTAT ACATCATGGC

2001 AGACAAACAA AAGAATGGAA TCAAAGTTAA CTTCAAAATT AGACACAACA

2051 TTGAAGATGG AAGCGTTCAA CTAGCAGACC ATTATCAACA AAATACTCCA

2101 ATTGGCGATG GCCCTGTCCT TTTACCAGAC AACCATTACC TGTCCACACA

2151 ATCTGCCCTT TCGAAAGATC CCAACGAAAA GAGAGACCAC ATGGTCCTTC

2201 TTGAGTTTGT AACAGCTGCT GGGATTACAC ATGGCATGGA TGAACTATAC

2251 AAATAGCATT CGTAGAATTC TATTGCACGG TATATGGCGA AGAAACATCA

2301 TATGATGGGT GAAACAGACG AGGAATACTA TAGTGTTGAA AAGTTGATTG

2351 GTCAGGTGAG TTGAGCCTTT TATTGGTTGA TGGGATTTTA TGCTACTGGA

2401 GTCCTGTAAG TTCAGTTTGT TGCGTTGAAT AAATAAAGTT CGAATTGATA

2451 GGCGAGTGGT AACCTATGTG TTGTGATTAC TAAAAGTCAT AAGTGTGAGG

2501 TATCCCAATT ATTAGAAATG TGACTAAGAC TGAGCTTTTC TGCCTATTGG

2551 GATATTACAG ATAAGTTACT TATTTGTAGT ATCGTAATAG TGTTCACTGG

2601 GAACCATCTA ATTCACTCTC AAAAATACTT TATCGTGTCT TATTTTATTC

2651 ATCCTCATAA CTCGTTTGCT AGGAGAACAG CTGTGCCCAT AGGTTAGTTG

2701 ACAAGTTACT TACTTCCCCA TGATACGCCT TATTTTAATA ACCTAATAAA

2751 GGCTCGGTTA CGTAACGTCC CTATAGTGCA TAGCATACCT ACGAACATTG

2801 ACTCCCAACT GATTAAGTGC GGATTGCATT TTTTTGGATG TTATCGCACA

2851 GTAAGACAAT ACCATCCTCA TCTCAATGTC AAGAGGTCTT TCTCCAGGCA

Fig. 6 continued

2901 ACAGATCCAT ACCACCATTA CTTACATCCA TCAGAGCTGT TCCCAGAATG
2951 TCTTGTTATA AACGGATGGA TTATTTATTT ATTTGACCAC ATAAATATTG
3001 CATCAAAGAG GTGGGGGATC CACTAGTTCT AGAGCGGCCG CCACCGCGGT
3051 GGAGGATCTG AATCCGACCA ACCGTTCTAT GAAAATCGTT GTATCTCCGA
3101 AACCACTGGA CGGATTTTTA TGATGTTTGT TTTAGATTAT TTGCGAGAGC
3151 GTGGGCGTTA ATATAAAACA AGAATGATCT GAATCCGACC AACCGTTCTA
3201 TGAAAATCGT TGTATCTCCG AAACCACTGG ACGGATTTTT ATGATGTTTG
3251 TTTTAGATTA TTTGCGAGAG CGTGGGCGTT AATATAAAAC AAGAATGATC
3301 TGAATCCGAC CAACCGTTCT ATGAAAATCG TTGTATCTCC GAAACCACTG
3351 GACGGATTTT TATGATGTTT GTTTTAGATT ATTTGCGAGA GCGTGGGCGT
3401 TAATATAAAA CAAGAATCAT CTCAATCCCA TCAGCCGTTC TATGAAAATC
3451 GTTGTATCTC CGAAACCACT GGACGGATTT TTATGATGTT TGTTTTAGAT
3501 TATTTGCGAG AGCGTGGGCG TTAATATAAA ACAAGAATGA TCTGAACACG
3551 GGTGTTTTTC TGTTCAGCTT ATGCAACTTT AAAATTCGAT GGGTCGTCTC
3601 AACGAAATTT GTATTGCTTT GCTCCAGCTT TTGTTCCCTT TAGTGAGGGT
3651 TAATTGCGCG CTTGGCGTAA TCATGGTCAT AGCTGTTTCC TGTGTGAAAT
3701 TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGGAA GCATAAAGTG
3751 TAAAGCCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC
3801 GCTCACTGCC CGCTTTCCAG TCGGGAAACC TGTCGTGCCA GCTGCATTAA
3851 TGAATCGGCC AACGCGCGGG GAGAGGCGGT TTGCGTATTG GGCGCTCTTC
3901 CGCTTCCTCG CTCACTGACT CGCTGCGCTC GGTCGTTCGG CTGCGGCGAG
3951 CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG
4001 GATAACGCAG GAAAGAACAT GTGAGCAAAA GGCCAGCAAA AGGCCAGGAA
4051 CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG
4101 ACGAGCATCA CAAAAATCGA CGCTCAAGTC AGAGGTGGCG AAACCCGACAG
4151 GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT
4201 CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC
4251 GGGAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT CTCAGTTCGG
4301 TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGACCC CCCGTTCAGC
4351 CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA
4401 AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA GGATTAGCAG
4451 AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG TGGCCTAACT

Fig. 6 continued

4501 ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA
4551 GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC
4601 CGCTGGTAGC GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA
4651 AAAAAGGATC TCAAGAAGAT CCTTTGATCT TTTCTACGGG GTCTGACGCT
4701 CAGTGGAACG AAAACTCACG TTAAGGGATT TTGGTCATGA GATTATCAAA
4751 AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT TTTAAATCAA
4801 TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC
4851 AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT CCATAGTTGC
4901 CTGACTCCCC GTCGTGTAGA TACTACGATA CGGGAGGGCT TACCATCTGG
4951 CCCCAGTGCT GCAATGATAC CGCGAGACCC ACGCTCACCG GCTCCAGATT
5001 TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG AAGTGGTCCT
5051 GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG
5101 AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTA
5151 CAGGCATCGT GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC
5201 GGTTCCCAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA
5251 AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAAGT AAGTTGGCCG
5301 CAGTGTTATC ACTCATGGTT ATGGCAGCAC TGCATAATTC TCTTACTGTC
5351 ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC
5401 ATTCTGAGAA TAGTGTATGC GGCGACCGAG TTGCTCTTGC CCGGCGTCAA
5451 TACGGGATAA TACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT
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5551 ATCCAGTTCG ATGTAACCCA CTCGTGCACC CAACTGATCT TCAGCATCTT
5601 TTACTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG GCAAAATGCC
5651 GCAAAAAAGG GAATAAGGGC GACACGGAAA TGTTGAATAC TCATACTCTT
5701 CCTTTTTCAA TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG
5751 GATACATATT TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCCGCGC
5801 ACATTTCCCC GAAAAGTGCC ACCTGACGCG CCCTGTAGCG GCGCATTAAG
5851 CGCGGCGGGT GTGGTGGTTA CGCGCAGCGT GACCGCTACA CTTGCCAGCG
5901 CCCTAGCGCC CGCTCCTTTC GCTTTCTTCC CTTCCTTTCT CGCCACGTTC
5951 GCCGGCTTTC CCCGTCAAGC TCTAAATCGG GGGCTCCCTT TAGGGTTCCG
6001 ATTTAGTGCT TTACGGCACC TCGACCCCAA AAAACTTGAT TAGGGTGATG

Fig. 6 continued

6051 GTTCACGTAG TGGGCCATCG CCCTGATAGA CGGTTTTTCG CCCTTTGACG

6101 TTGGAGTCCA CGTTCTTTAA TAGTGGACTC TTGTTCCAAA CTGGAACAAC

6151 ACTCAACCCT ATCTCGGTCT ATTCTTTTGA TTTATAAGGG ATTTTGCCGA

6201 TTTCGGCCTA TTGGTTAAAA AATGAGCTGA TTTAACAAAA ATTTAACGCG

6251 AATTTTAACA AAATATTAAC GCTTACAATT TCCATTCGCC ATTCAGGCTG

6301 CGCAACTGTT GGGAAGGGCG ATCGGTGCGG GCCTCTTCGC TATTACGCCA

6351 GCTGGCGAAA GGGGGATGTG CTGCAAGGCG ATTAAGTTGG GTAACGCCAG

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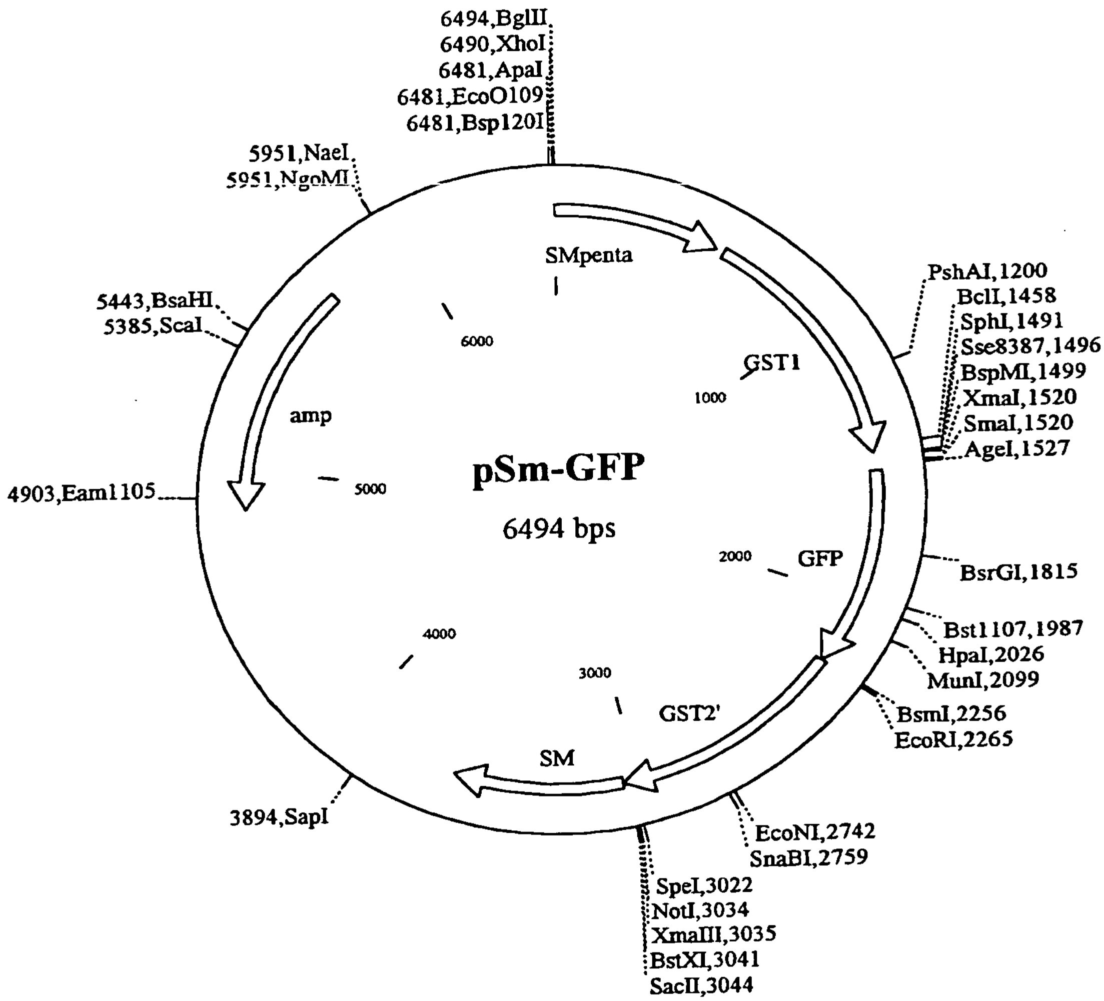
Figure 7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MunI,2099
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BsmI,2256
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SM
3894,SapI
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XmaIII,3035
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SacII,3044

Figure 5

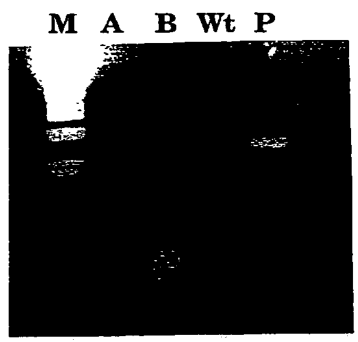

Fig 9a Fig 9b

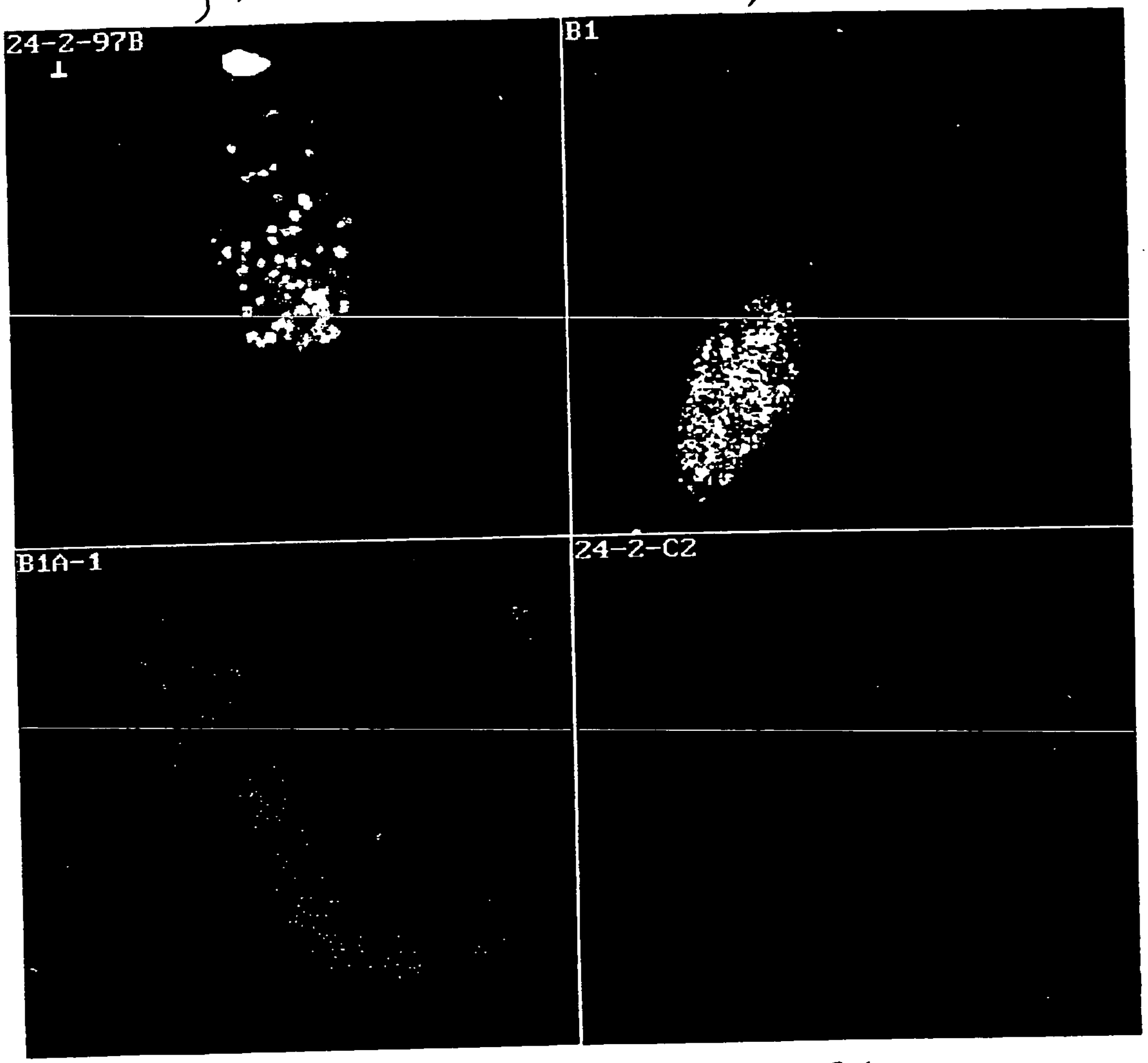

Fig 9c Fig 9d

Figure 7

Fig 10a Fig 10b

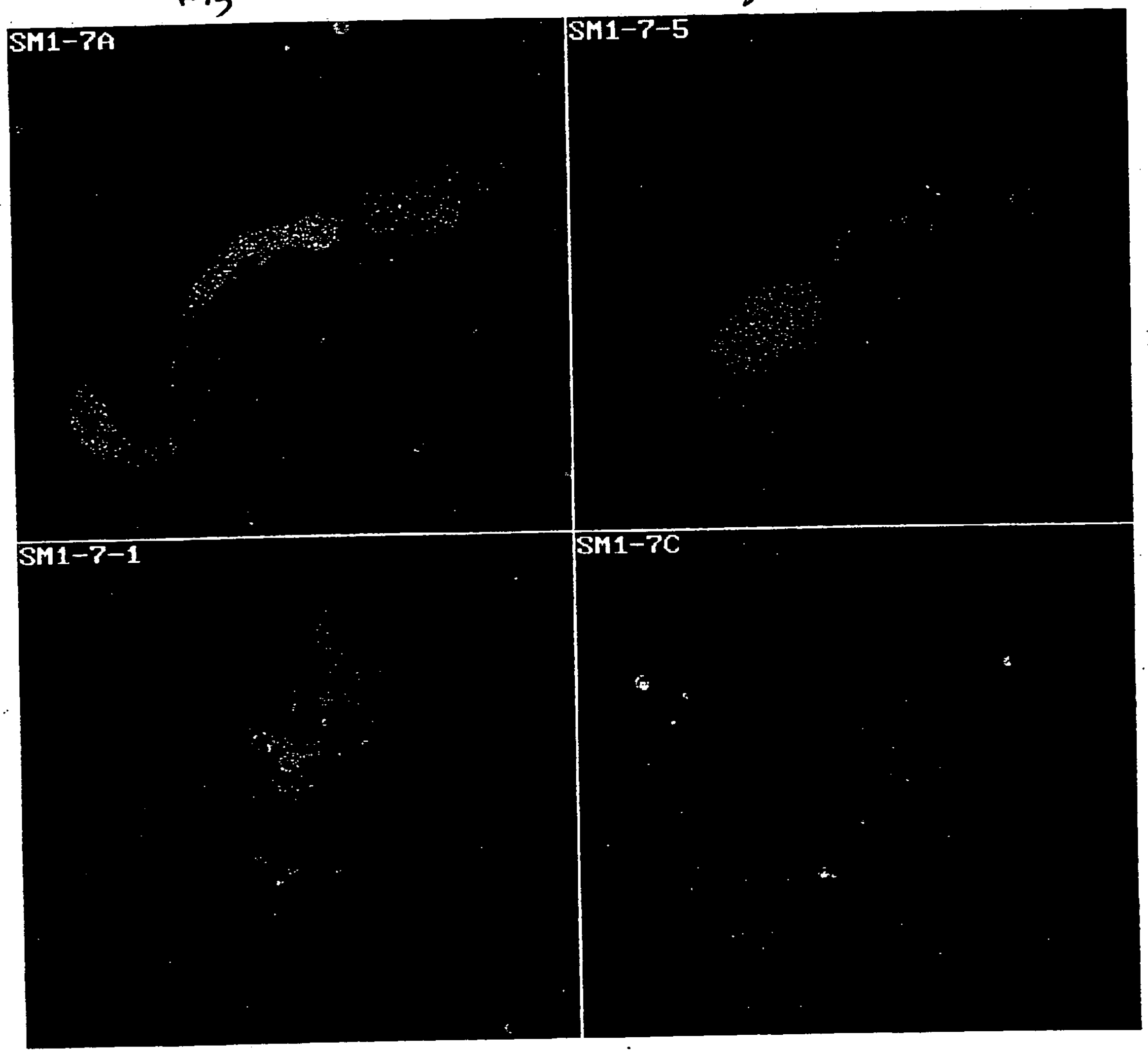

Fig 10c Fig 10d

Fig 11a Fig 11b Fig 11c

Fig 11d

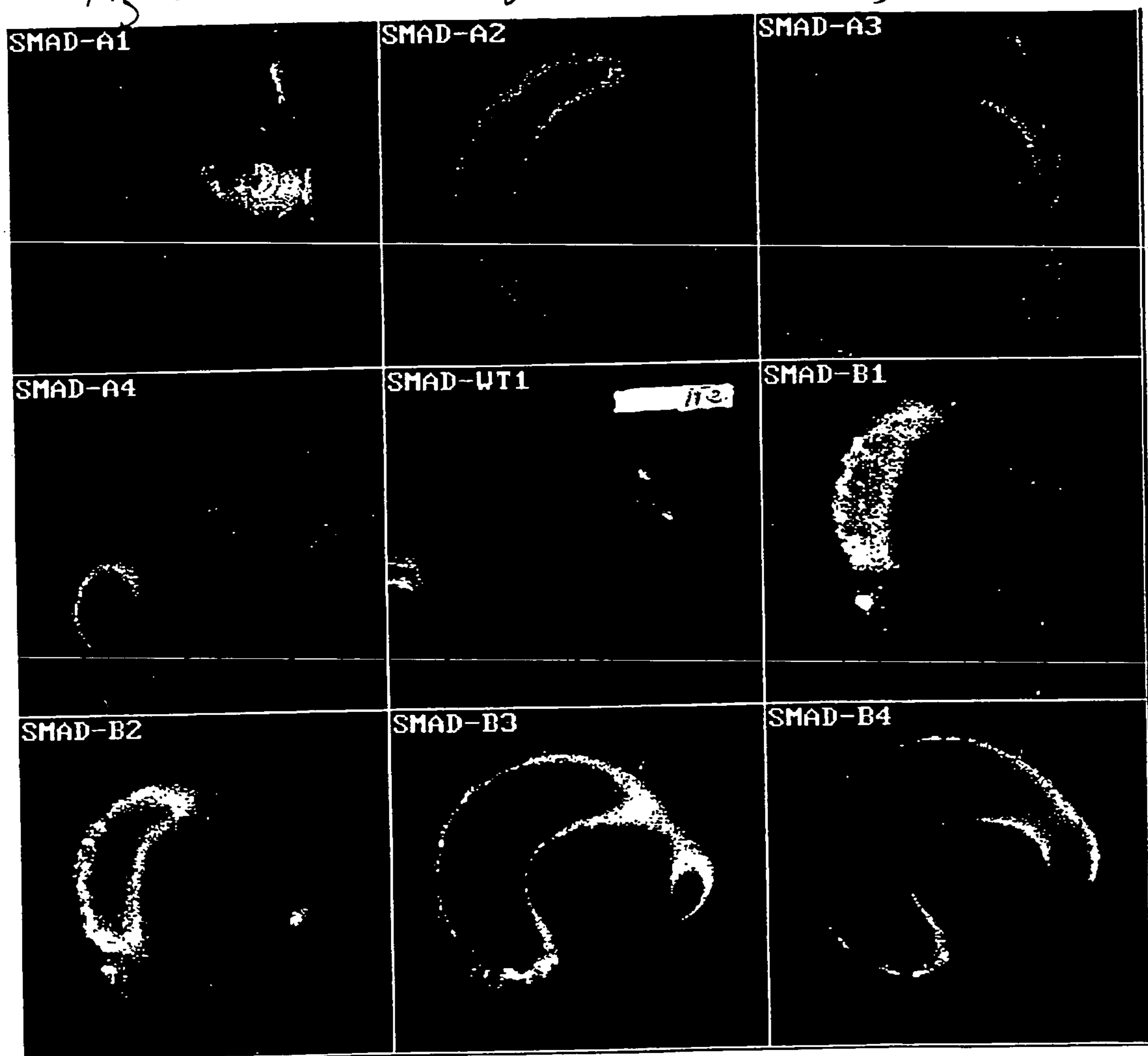

Fig 11f

Fig 11g Fig 11h Fig 11i

Fig 12a Fig 12b Fig 12c

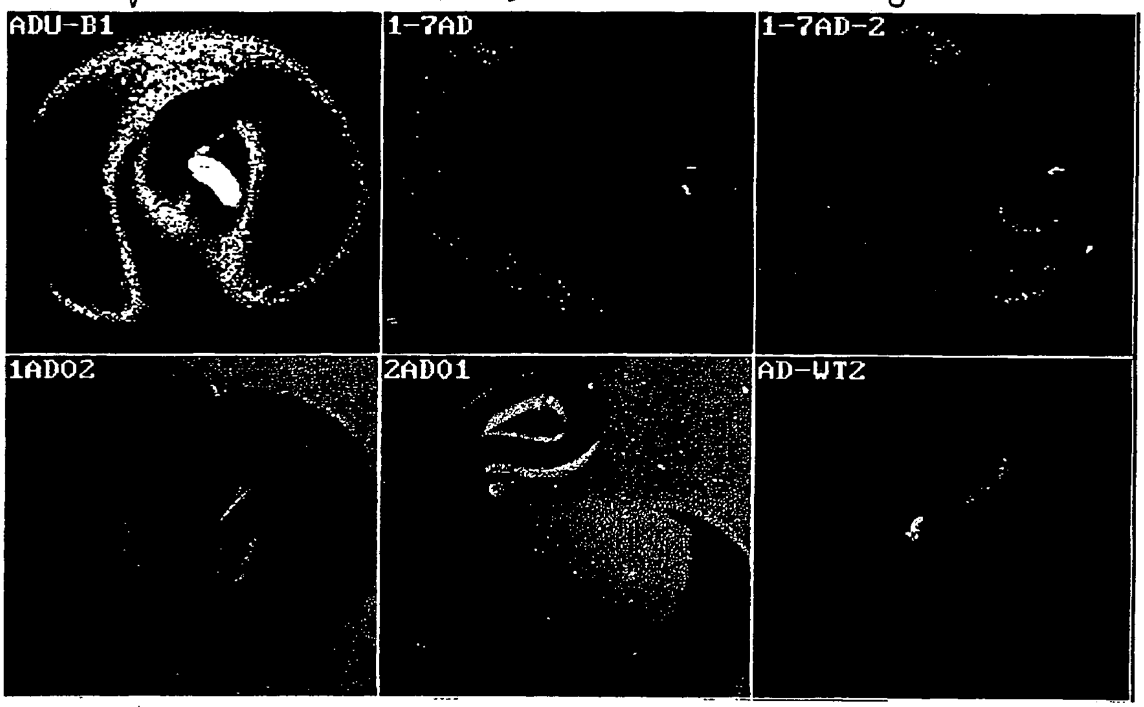

Fig 12d Fig 12e Fig 12f

Fig 13a

Fig 13b

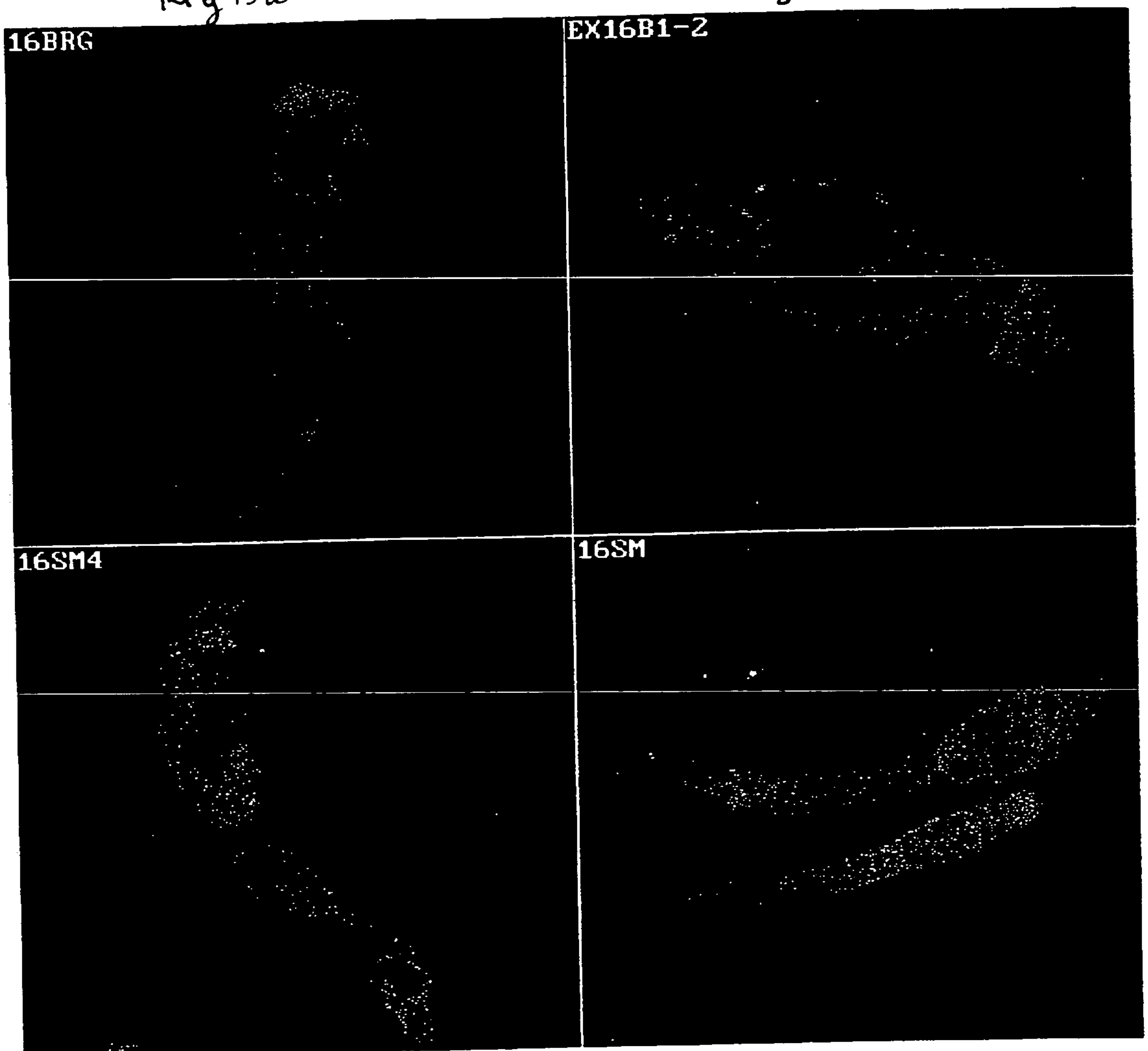

Fig 13c

Fig 13d

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